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XUM

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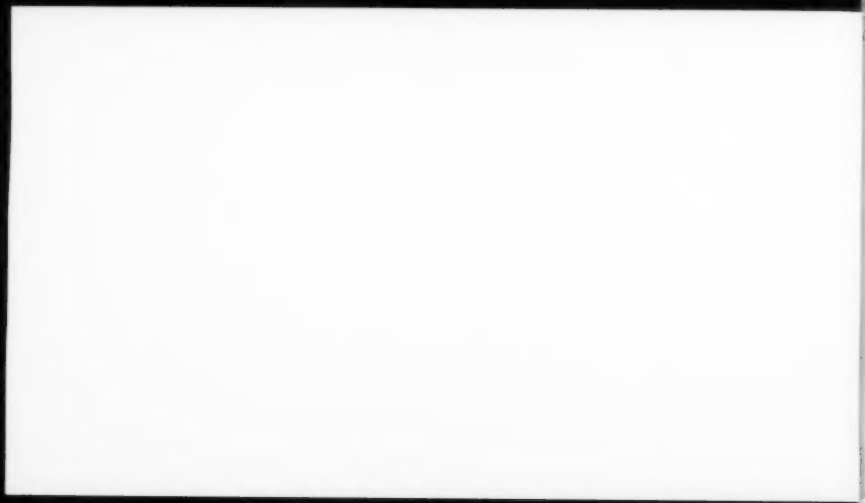
CORRECTIONS.

On page 567, Vol. lxx, No. 2, October, 1926, last column of Table IV, read:

Non-protein CO_2	0.771 gm.,	instead of	0.742 gm.
" O_2	0.626 "	" "	0.600 "
" R. Q.	0.895	" "	0.899
Fat oxidized	0.078 gm.,	" "	0.072 gm.
Calories from fat	0.73	" "	0.68
Total calories	2.45	" "	2.40



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THE CHEMICAL STUDIES OF THE OVARY.*

XI. THE FAT OF OVARIAN RESIDUE.

By DEE TOURTELLOTTE† and MERRILL C. HART.

(From the Research Laboratories, The Upjohn Company, Kalamazoo.)

(Received for publication, September 30, 1926.)

Different structures physiologically such as the corpus luteum and ovarian residue would naturally be expected to reflect this difference in their chemical composition. We have been particularly interested in comparing the lipoid constituents of these two tissues. The phosphatide content of corpus luteum[1,2] was considerably greater than that of ovarian residue. From corpus luteum we isolated 1.2 per cent of an ether-soluble, acetone-, and alcohol-insoluble (cephalin) fraction, and 5.8 per cent of lecithin cadmium chloride. Similar fractions from ovarian residue amounted to only 0.3 and 0.03 per cent respectively. Possibly also, some or all of the phosphatide found in the ovarian residue might be due to the inability to separate completely the corpus luteum from the ovarian residue. Cholesterol also was present in greater amount (1.3 per cent) in corpus luteum than in ovarian residue (0.5 per cent). Ovarian residue, however, contains a larger amount of protagon-like material. From ovarian residue we obtained 1.2 per cent of this ether-insoluble material and from corpus luteum 0.1 per cent.

* The general heading of this series of papers has been changed to "The Chemical Studies of the Ovary." Previous papers in this series have been published under the separate titles of, "The Chemical Investigations of Corpus Luteum," "The Chemical Examination of Ovarian Residue," and, "The Chemical Composition and Biological Activity of Liquor Folliculi."

† Holder of the Upjohn Cooperative Fellowship at Kalamazoo College (1925-1926). This paper is based upon the thesis presented by Mr. Tourtellotte to the Faculty of Kalamazoo College, in partial fulfilment of the requirements for the degree of Master of Science.

[1] Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1925, lxvi, 639.

[2] Hart, M. C., and Heyl, F. W., *J. Biol. Chem.*, 1926, lxx, 663.

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We were particularly interested in this paper in comparing the fat of ovarian residue with the fat of corpus luteum. The dried corpus luteum studied contained 13.1 per cent of fat with an iodine number of 78. We have shown in a previous publication,[3] that this fat contained 35 per cent of saturated acids and 65 per cent unsaturated. The fatty acids were 25 per cent palmitic, 11.2 per cent stearic, 1.6 per cent ($C_{16}H_{30}O_2$), 32.8 per cent oleic, 16.6 per cent linolic, 8 per cent arachidonic, and 4.8 per cent of a new tri unsaturated acid of the C_{20} series having the formula $C_{20}H_{34}O_2$. The fat of ovarian residue was found on examination to contain more palmitic acid (32.4 per cent) and approximately the same amount of stearic. Myristic acid was also probably present to the extent of 0.4 per cent. This is of interest in relation to the finding of Eckstein[4] of approximately 1 per cent of myristic in adipose tissue fat. Oleic acid is present in considerably larger amounts (55.3 per cent) in ovarian residue than in the corpus luteum. Arachidonic acid and the tri unsaturated acid are present in smaller amounts than found in corpus luteum. Slightly more arachidonic acid was found than Eckstein[4] showed to be present in depot fat. Linolic acid, however, was present only in traces.

EXPERIMENTAL.

6.13 kilos of fat-free ovarian residue (cows) was ground to pass through a No. 20 sieve and exhaustively extracted fractionally with ether, 100 liters being used. The material used in the following investigation represents the acetone-soluble part of this ether extract. A total solid determination showed the presence of 264 gm. of fat.

The ether solution of the fat was extracted with water and 1 per cent sulfuric acid to remove any water-soluble nitrogen bases and inorganic material. The water extract contained 1.270 gm. of solid material which contained 6.12 per cent N and 1.48 per cent P. The 1 per cent sulfuric acid extract analyzed for 0.0688 gm. of total nitrogen and 0.0129 gm. of total phosphorus.

The purified fat weighed 261 gm. This contained 0.41 per cent N and 0.19 per cent P. For preliminary analysis, 30 gm. of the purified fat were dried to constant weight and the following

[3] Cartland, G. F., and Hart, M. C., *J. Biol. Chem.*, 1925, lxvi, 619.
[4] Eckstein, H. C., *J. Biol. Chem.*, 1925, lxiv, 797.

determinations made. Where possible official methods as given in Bulletin 107, United States Department of Agriculture, were used; otherwise Lewkowitsch[5] was followed:

Specific gravity, $\frac{100°}{100°}$ C.....	0.9066	Reichert-Wollny value..	1.36
		Unsaponifiable.....	18.8
		per cent.	
Iodine No. (Hanus)..	69.54	Cholesterol[6].....	12.4
Acid value.....	67.58	Acetyl value.....	41.2
Saponification value.	120.8		

The free fatty acids were separated from 247 gm. of fat by extraction with 10 per cent potassium hydroxide. The ether solution of the neutral fat was taken to dryness and saponified by refluxing for 10 hours with 10 per cent alcoholic potassium hydroxide. The alcohol was removed by distillation[7] and the soap dissolved in water. The unsaponifiable was then removed by extraction with ether. The acids were recovered from the alkaline soap solutions by acidifying with 20 per cent hydrochloric acid and extracting the liberated fatty acids with ether.

By this procedure the 247 gm. of fat were separated into the following three fractions:

1. 100 gm. of free fatty acids.
2. 95 " " fatty acids from neutral fat.
3. 46.3 " " unsaponifiable material.

The cholesterol was separated from the unsaponifiable material by crystallization from alcohol. 30.76 gm. of cholesterol were obtained, melting at 148–149°C. The unsaponifiable residue remaining after separating the cholesterol was stored under nitrogen for future physiological and chemical examination.

Free Fatty Acids.

The 100 gm. of free fatty acids in ether solution were separated into three fractions by extraction with varying strengths of

[5] Lewkowitsch, J., Chemical technology and analysis of oils, fats and waxes, London, 6th edition, 1921, i.

[6] Method of MacArthur (MacArthur, C. G., *J. Am. Chem. Soc.*, 1919, xli, 1232).

[7] All distillations were conducted at reduced pressure in an atmosphere of nitrogen.

alkaline solutions. The fatty acids were recovered from the alkaline extracts by acidifying with hydrochloric acid and extracting the liberated acids with ether. The following fractions were obtained:

10 per cent	ammonium carbonate extract,	1.8 gm.	acids.
10 " "	sodium " "	85.6 "	"
10 " "	potassium hydroxide "	13.8 "	"

Ammonium Carbonate Extract of Free Fatty Acids.

This extract (1.8 gm.) consisted of a light colored oil which gave a very strong odor of volatile fatty acids. Nothing crystalline was obtained upon allowing the alcoholic solution to stand at various concentrations in the ice box.

The oil was mixed with 10 per cent sulfuric acid and steam-distilled. The total volatile acids in the distillate were equivalent to 9.63 cc. of normal acid. The neutral solution was concentrated to 100 cc., acidified, and the volatile acids extracted with ether. Ammonia water was added to the ether solution and the ether evaporated. The excess ammonia was removed by boiling and the volatile acids precipitated as silver salts. Three fractions of silver salts were obtained which were recrystallized from water, filtered, washed, dried, and analyzed for silver.

Analyses.

Fraction 1. 0.0732 gm. substance: Ag 0.0377 gm.
Found. Ag 51.5.

Fraction 2. 0.0999 gm. substance: Ag 0.0537 gm.
Found. Ag 53.8.

Fraction 3. 0.1325 gm. substance: Ag 0.0716 gm.
Found. Ag 54.0.
Calculated for $C_4H_7O_2Ag$. Ag 55.3.
" " $C_6H_{11}O_2Ag$. " 48.4.

These results suggest that the volatile acids consist of a mixture of butyric and caproic acids.

Sodium Carbonate Extract of Free Fatty Acids.

The sodium carbonate extract consisting of 85.6 gm. of acids was dissolved in 95 per cent alcohol and cooled in the ice box.

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A quantity of solid acids separated which upon recrystallization from alcohol gave 17.15 gm. of crystalline acids, melting at 56–58°C.

The alcoholic mother liquor from the above crystallization was evaporated to dryness and the residual acids weighing 68.4 gm. distilled *in vacuo*. These acids distilled at 180–220°C. at 2.5 mm. pressure. On crystallization of this distillate, 5.75 gm. of acids melting at 52–54°C. were obtained. The alcoholic mother liquor from this crystallization was evaporated to dryness and the remaining acids, 47.39 gm., were separated into three fractions by the barium-lead salt process.[8] By this means the total fatty acids from the sodium carbonate extract were separated into the following three fractions:

1. 18.46 gm. of unsaturated acids, iodine No. 118.
2. 18.47 " " intermediate fraction, iodine No. 90.
3. 29.29 " " saturated acids, iodine No. 18.

These fractions were later joined for analysis to like fractions of acids from the neutral fat.

Potassium Hydroxide Extract of Free Fatty Acids.

These acids (13.8 gm.) were crystallized from alcohol. 2.28 gm. of crystalline acids melting at 54–56°C. were obtained. The mother liquors from this crystallization yielded 11.5 gm. of acids on evaporation of the solvent. These acids were distilled *in vacuo* at 1.5 mm. pressure. 9.70 gm. of an amber-colored oil which solidified on cooling were obtained. This on crystallization from alcohol gave 1.10 gm. more of crystalline fatty acids melting at 61–63°C. These crystalline acids were examined in conjunction with the other solid acids and the remainder of the fatty acids was separated into the following three fractions by the barium-lead salt process:

1. 2.78 gm. of unsaturated acids, iodine No. 156.
2. 4.58 " " intermediate fraction, iodine No. 100.
3. 0.75 " " saturated acids, iodine No. 17.

These two unsaturated fractions were further studied by the formation of their bromine derivatives by the method of Baugh-

[8] Levene, P. A., and Rolf, I. P., *J. Biol. Chem.*, 1922, li, 509.

man and Jamieson.[9] 4.58 gm. of the intermediate fraction gave 0.11 gm. of ether-insoluble bromides decomposing at 224°C.

This fraction was separated by extraction with boiling benzene in a Soxhlet apparatus into 0.05 gm. of benzene-insoluble bromide decomposing at 226°C. and 0.05 gm. of benzene-soluble material decomposing at 220°C. There was insufficient material for analysis but the decomposition points indicate impure arachidonic octobromide. The petroleum ether-insoluble bromides amounted to 0.01 gm. and decomposed at 170–180°C. The ether- and petroleum ether-soluble dibromides weighed 6.31 gm. This fraction was reduced with copper-coated zinc by the method of Wesson.[10] 3.45 gm. of acid with an iodine number of 90 were obtained. 2.0 gm. of this acid were reduced with hydrogen and colloidal palladium by the method of Paal. The reduced solid acid on several crystallizations from alcohol gave 0.44 gm. of crystalline acid melting at 68°C. A mixed melting point with stearic acid was not depressed. This was dried to constant weight *in vacuo* at the temperature of boiling toluene and analyzed.

Analysis.

0.1482 gm. substance: CO_2 0.4138, H_2O 0.1689.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
Found. " 76.1, " 12.7.

The study of this fraction by means of this bromination method indicates that this fraction consists largely of oleic acid contaminated with small amounts of the more highly unsaturated acids.

2.45 gm. of the unsaturated acids were also brominated. The washed and dried, ether-insoluble bromides weighed 0.64 gm. and decomposed at 224°C. 0.62 gm. of this on extraction with boiling benzene in a Soxhlet apparatus gave 0.31 gm. of benzene-insoluble bromide decomposing at 226°C. This was dried to constant weight *in vacuo* at the temperature of boiling toluene and the bromine determined by the Carius method.

[9] Baughman, W. F., and Jamieson, G. S., *J. Am. Chem. Soc.*, 1922, xlii, 2947.

[10] Wesson, L. G., *J. Biol. Chem.*, 1924, lx, 183.

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Analysis.

0.2043 gm. substance: AgBr 0.3188.
Calculated for $C_{20}H_{32}O_2Br_8$. Br 67.8.
Found. " 66.4.

These results indicate that this material is mainly arachidonic octobromide.

The benzene extract yielded 0.31 gm. of a bromide which blackened and decomposed at 220°C. This was dried to constant weight and analyzed for bromine.

Analysis.

0.1601 gm. substance: AgBr 0.2398.
Calculated for $C_{18}H_{36}O_2Br_6$. Br 63.3.
" " $C_{20}H_{34}O_2Br_6$. " 61.0.
Found. " 63.7.

This analysis suggests linolenic hexabromide. Linolenic hexabromide, however, melts at 181°C. This fraction probably consists of a hexabromide of the C_{20} series contaminated with some octobromide. We have found indications for this same hexabromide in the fat of corpus luteum as well as in the fatty acids from the hydrolysis[2] of the lecithin from corpus luteum.

Traces only of a petroleum ether-insoluble fraction were obtained. The soluble dibromide fraction amounted to 4.02 gm. On removal of the bromine from this, 1.65 gm. of acid with an iodine number of 90 were obtained. This acid was reduced with hydrogen and colloidal palladium. The solid acid after several crystallizations from alcohol melted at 62°C. This was dried to constant weight in the usual manner and analyzed.

Analysis.

0.1439 gm. substance: CO_2 0.4013, H_2O 0.1635.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
Found. " 76.0, " 12.7.

The study of this fraction indicates that it consists largely of oleic acid with larger amounts of the more unsaturated acids, such as arachidonic and the tri unsaturated acid of the C_{20} series, than the intermediate fraction reported above. Linolenic acid appears to be absent and no evidence of the presence of linolic acid was found.

Fatty Acids of Neutral Fat.

These acids weighing 95 gm. were dissolved in ether and extracted with varying strengths of alkaline solutions. The following fractions were obtained:

10 per cent ammonium carbonate, 21.0 gm. acids.
10 " " sodium " 73.5 " "
10 " " potassium hydroxide, 0.35 " "

Examination of Ammonium Carbonate Extract.

This extract on crystallization from alcohol gave 4.36 gm. of crystalline acids, melting at 52–57°C. These were examined along with the other saturated acids. The rest of the acid in this fraction was distilled *in vacuo*. On crystallization of the distillate from alcohol, 0.85 gm. more of solid acid melting at 54–56°C. was obtained. The remainder of the acids (9.88 gm.) was separated by the barium-lead salt process into the following fractions:

1. 1.69 gm. of unsaturated acids, iodine No. 120.
2. 6.38 " " intermediate fraction, iodine No. 95.
3. 1.12 " " saturated acids, iodine No. 29.

1.40 gm. of the unsaturated fraction were brominated. The ether-insoluble bromides weighed 0.16 gm. The benzene-insoluble part of this weighed 0.06 gm. and decomposed at 230°C. The benzene-soluble part weighed 0.09 gm. and decomposed at 216°C. A very small amount (0.04 gm.) of petroleum ether-insoluble bromides was obtained. This was a bad mixture as it melted and decomposed at 150–180°C. The petroleum ether-soluble bromides (2.35 gm.) were debrominated and the recovered acid (0.94 gm.) gave an iodine number of 108. 0.62 gm. was reduced and after crystallization from alcohol 0.082 gm. of acid melting at 66–67°C. was obtained. This was dried to constant weight and analyzed.

Analysis.
0.0817 gm. substance: CO_2 0.2282, H_2O 0.0948.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
Found. " 76.1, H 12.9.

The analysis of this fraction indicates oleic acid as well as smaller amounts of arachidonic and this tri unsaturated acid of the C_{20} series.

Examination of Sodium Carbonate Extract.

This extract on crystallization from alcohol gave 23.7 gm. of crystalline acids melting at 48–56°C. The acids from the alcohol

TABLE I.

Distillation of Unsaturated Acids.

Fraction.	Weight.	Boiling point.	Pressure.	Iodine No.	Neutralization value.*	Molecular weight.
	gm.	*°C.*	*mm.Hg*			
1	4.02	140–185	0.6	90	121	480
2	6.28	185–200	0.6	120	153	366
3	4.61	200–210	0.6	137	173	324
4	6.36	210–235	0.6	188	168	334

* The neutralization values were abnormally low due to some material which was not readily saponified by N/10 sodium hydroxide.

TABLE II.

Bromination of Unsaturated Acids.

Fraction.	Acid taken.	Ether-insoluble bromides.	Tetrabromide.	Dibromide.	Acid from dibromide.	Iodine No. Acid from dibromide.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	
1	2.74	0.10	0.034	4.08	0.87	86
2	5.79	0.44	0.125	9.06	4.42	103
3	4.23	0.54	0.078	7.30	3.73	103
4	5.89	1.79	0.259	10.35	4.88	140

filtrate were distilled *in vacuo* and the distillate (44.84 gm.) was separated by the barium-lead salt process into three fractions.

1. 9.73 gm. of unsaturated acids, iodine No. 137.
2. 25.89 " " intermediate fraction, iodine No. 88.
3. 5.56 " " saturated acids, iodine No. 27.

Examination of Unsaturated Acids.

These acids were joined with a similar fraction from the sodium carbonate extract of the free fatty acids and fractionally distilled

at a pressure of 0.6 mm. Four fractions were obtained. Neutralization values and iodine numbers of the fractions were determined and their mean molecular weights determined. These results are summarized in Table I.

Each of the four fractions from the above distillation was studied by the formation of the bromides. The results of this bromination are summarized in Table II.

The ether-insoluble bromides from the above bromination blackened and decomposed at 226–230°C. Fractions 3 and 4 of the ether-insoluble bromides were joined and separated by extraction in a Soxhlet apparatus with boiling benzene into the benzene-soluble and benzene-insoluble fractions. The benzene-insoluble fraction (1.12 gm.) decomposed at 231°C. This was dried and analyzed for bromine.

Analysis.

0.1417 gm. substance: AgBr 0.2224.
Calculated for $C_{20}H_{32}O_2Br_8$. Br. 67.8.
Found. " 66.8.

The bromine determination, solubilities, and decomposition point indicate arachidonic octobromide.

The benzene-soluble fraction weighed 1.15 gm. This decomposed at 220°C. It was analyzed.

Analysis.

0.1613 gm. substance: AgBr. 0.2421.
Calculated for $C_{20}H_{34}O_2Br_6$. Br 61.0.
" " $C_{18}H_{30}O_2Br_8$. " 63.3.
Found. " 63.9.

This benzene-soluble bromide represents a mixture of a hexabromide of the C_{20} series and arachidonic octobromide.

From the petroleum ether-insoluble bromides a trace of linolic tetrabromide was found. These bromides were joined, dissolved in cold ether, and separated from traces of ether-insoluble material. The ether was removed and the residue crystallized from petrolic ether. 0.035 gm. of crystalline tetrabromide melting at 111–112°C. was obtained.

The acids obtained by debrominating the petroleum ether-soluble fraction represent the larger part of the unsaturated acids (Table II). The high iodine number of Fraction 4 was due to

incomplete bromination. On rebromination this fraction yielded 0.37 gm. more of ether-insoluble bromide which decomposed at 226–228°C. Fractions 2 and 3 were joined and an aliquot taken for reduction with hydrogen. 2.5 gm. of acid were reduced and after several crystallizations from alcohol 0.67 gm. of acid melting at 67–68°C. was obtained. This was dried to constant weight and analyzed.

Analysis.

0.1472 gm. substance: CO_2 0.4098, H_2O 0.1662.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
Found. " 75.9, " 12.6.

The acid in this dibromide fraction is oleic.

TABLE III.

Distillation of Intermediate Fractions.

Fraction.	Weight.	Boiling point.	Pressure.	Iodine No. (Hanus).
	gm.	*°C.*	*mm.Hg*	
1	1.12	145-185	1.3	74
2	19.39	185-195	1.4	85
3	18.64	195-198	1.2	98

Examination of Intermediate Fraction of Unsaturated Acids.

This fraction represents the acids whose barium salts were insoluble in the benzene-ether mixture and whose lead salts were ether-soluble. The intermediate fraction (iodine No. 90) from the sodium carbonate extract of the free fatty acids was joined with this (iodine No. 88) from the sodium carbonate extract of the fatty acids from the neutral fat, and the total acids (44.16 gm.) fractionally distilled at 1.3 mm. pressure. The results of the distillation are summarized in Table III.

2.5 gm. of Fraction 3 were reduced with hydrogen and colloidal palladium. There was obtained after crystallization 0.90 gm. of acid melting at 69–70°C. This was dried and analyzed.

Analysis.

0.1452 gm. substance: CO_2 0.4063, H_2O 0.1679.
Calculated for $C_{18}H_{36}O_2$. C 76.0, H 12.7.
Found. " 76.3, " 12.9.

These results are interpreted to indicate that these intermediate fractions from the barium-lead salt separation consist largely of oleic acid. Fraction 3 was probably contaminated with small amounts of arachidonic acid as shown by its iodine number and by the slightly high figures obtained on the analysis of the reduced acid.

TABLE IV.

Distillation of Methyl Esters of Saturated Acids.

Fraction.	Temperature.	Pressure.	Ester.	Acid from ester.	Acid after crystallization.	Melting point.
	°C.	mm.Hg	gm.	gm.	gm.	°C.
1	145–150	0.75	5.58	5.15	2.0	56
2	150–160	0.7	5.15	4.75	1.8	56–57
3	160–165	0.6	10.71	9.9	4.25	57
4	165–170	0.6	15.90	15.1	4.6	60–61
5	170–175	0.6	12.10	11.5	4.14	68–69

TABLE V.

Analysis of Saturated Acids.

Fraction.	Neutralization value.	Analysis.				
		Substance.	CO_2	H_2O	C	H
		gm.	gm.	gm.	per cent	per cent
1	221	0.1462	0.3997	0.1610	74.6	12.3
2	219.1	0.1541	0.4224	0.1740	74.8	12.6
3	217	0.1571	0.4334	0.1777	75.2	12.7
4	208	0.1444	0.3981	0.1630	75.2	12.6
5	202	0.1540	0.4277	0.1755	75.7	12.7
Calculated for:						
$C_{18}H_{36}O_2$	197				76.0	12.7
$C_{16}H_{32}O_2$	219				75.0	12.5
$C_{14}H_{28}O_2$	246.1				73.7	12.3

Examination of Saturated Acids.

The saturated acids from the lead salt separations and those from the crystallization of the crude acid mixture were joined. These acids weighed 67 gm. After recrystallization from 95 per cent alcohol, 52.5 gm. of acids were obtained which melted at 55–56°C.

These acids were converted into the methyl esters by refluxing for 4 hours with 100 cc. of methyl alcohol and 5 cc. of sulfuric acid. 52.8 gm. of methyl esters were obtained which were fractionally distilled at a pressure of 0.7 mm. of mercury. Each of the fractions was saponified, the soap decomposed with dilute sulfuric acid, and the liberated acids extracted with ether. The ether solutions were washed free from sulfuric acid, dried over anhydrous sodium sulfate, and the ether removed. The resulting acids were weighed, crystallized thrice from alcohol, and the melting points determined. The results are given in Table IV.

The crystalline acids from the above distillation were analyzed for C and H and the neutralization values determined. From the analyses, the composition of the fatty acid mixture is calculated to be 75 per cent palmitic, 24 per cent stearic, and 1 per cent myristic acid. These results are tabulated in Table V.

SUMMARY.

247 gm. of the fat of ovarian residue freed from acetone-insoluble phosphatides yielded 195 gm. of fatty acids and 46.3 gm. of unsaponifiable material. From the unsaponifiable material 30.76 gm. of cholesterol were crystallized. 49 per cent of the fatty acids are present in glycerides and 51 per cent present as free acids. 43.3 per cent of the total fatty acids are saturated and 56.7 per cent are unsaturated. The experimental work presented in this paper indicates the following composition of the fatty acid mixture: 0.4 per cent myristic, 32.4 per cent palmitic, 10.4 per cent stearic, 55.3 per cent oleic, trace of linolic, 0.7 per cent arachidonic, and 0.7 per cent of a tri unsaturated acid of the C_{20} series having the formula $C_{20}H_{34}O_2$.

THE VALUE OF COCOA AND CHOCOLATE AS SOURCES OF PROTEIN IN THE DIET.

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The United States for many years has been the largest importer of cacao and cacao products, and since the war an enormous increase in such imports has occurred. Thus, for the period 1910 to 1914, the average yearly importation of cacao products was about 145 million pounds. In 1920, the importation was 345 million pounds, increasing in 1923 to 417 million pounds, at which level it has been approximately maintained to the present time.

Cocoa and chocolate are manufactured from the bean of the cacao tree, *Theobroma cacao*. The fruit of this tree when ripe consists of pods 7 to 10 inches long containing from 20 to 50 cocoa beans. At harvesting, the pods are opened and the pulp and seeds removed. The latter are then generally subjected to a fermentation process (1) involving the action of yeast as well as of bacteria in order to facilitate the removal of the pulp from the beans and to develop their characteristic color and aroma. Subsequent to this fermenting or "sweating," the beans are prepared for market by washing, drying, and occasionally by polishing. Probably the most important individual process in the manufacture of chocolate and cocoa relates to the roasting of the bean. The roasting is very carefully controlled, the temperatures employed ranging from 100–135°C. During this process the aroma of the bean is further developed and the husk is rendered readily removable from the remainder of the seed. After roasting the beans are cracked and the broken husk separated from the nibs by winnowing. During the process the germ is also removed by sieving. The nibs are ground to a fine powder, and, if they are to be used in the manufacture of chocolate, they are pressed into cakes after admixture with sugar and proper flavoring materials. If they are to be used in the manufacture of cocoa they are submitted to pressure in hydraulic presses which remove from 60 to 70 per cent of the cocoa butter. The original nibs contain from 45 to 50 per cent fat, while the cocoa powder contains from 25 to 35 per cent.

XUM

The average composition of cocoa as it is found on the American and English market is given in Table I. The nitrogenous compounds in cocoa have been investigated by Stutzer (2), who found in a sample of cocoa untreated by chemicals that theobromine accounted for 16.6 per cent of the total nitrogen, ammonia for 1.4, extractives for 6.3, and proteins for 75.9 per cent. The protein content of the cocoa was further subdivided on the basis of its solubility by animal enzymes. On this basis 44.6 per cent of the total nitrogen of the cocoa was present in the form of digestible protein and 31.3 per cent in the form of indigestible protein.

TABLE I.

Reported Average Analyses of Cocoa in America and England.

	American analyses.*	English analyses.†
Moisture	6.23	3.0–8.0
Protein	18.34	19.0–20.0 (N × 6.3)
Fat	26.69	26.0–31.0
Ash	5.49	3.9–8.8
Starch	11.14	2.7–7.3
Fiber	4.48	6.8–7.2
Theobromine	1.15	1.7–2.0
Caffeine	0.16	
Other N-free substances	26.32	29.0–31.0

* Reported by Winton, A. L., Bailey, E. M., and Silverman, M., in *Conn. Agric. Exp. Sta. 27th Ann. Rep.*, 1903, 125.

† Reported by an anonymous writer in Lancet, 1905, i, 47.

The solubility and digestibility of the nitrogenous compounds in cocoa are important considerations in any determination of the value of cocoa and chocolate as sources of protein in nutrition. In 1900 Forster (3) reported the results of chemical and physiological investigations of the nitrogen compounds of Dutch cocoa. Of the total nitrogen in this cocoa 44.5 per cent was soluble in water, 15.6 per cent was insoluble in water but was rendered soluble by artificial enzyme digestion, while 39.9 per cent was insoluble in water and indigestible by enzyme action. 5 years later an anonymous contributor in the Lancet (4) reported similar tests on English cocoas, the results of which indicated

that 33 per cent of the nitrogen of untreated cocoa was soluble in water and that 68 per cent was digested by pepsin and pancreatic juices.

The results of such tests as these indicate that a considerable fraction of the nitrogen of chocolate and cocoa exists in the nonprotein form, mainly as theobromine, and that of the protein in these products a large share cannot be brought into solution by animal enzymes. The indigestibility of much of the nitrogen of cocoa is further confirmed by animal experimentation. Schlesinger (5) cites digestion experiments by Weigmann upon himself showing that only 42 per cent of the nitrogen of cocoa is absorbed. In 1895 Cohn (6) also determined the digestibility of cocoa nitrogen by *in vitro* experiments as well as by digestion experiments on human subjects. He found that gastric and pancreatic digestion *in vitro* dissolved about 53 per cent of the nitrogen of cocoa powder. When cocoa was included in the mixed diet of human subjects, it was estimated that 53 per cent of its nitrogen remained undigested. Similar experimental results pointing to a low digestibility of cocoa nitrogen have been reported by Pincussohn (7) and by Neumann (8). In contrast to most of the published experimental data of this nature, Forster (3) cites human digestion experiments by Bruns which have been interpreted to mean that about 80 per cent of the nitrogen of cocoa is digested when consumed with milk.

In so far as the authors are aware, no experiments have been reported in the literature relating to the biological value of the nitrogen of chocolate and cocoa; *i.e.*, the maximum utilization of the absorbed nitrogen in metabolism. Furthermore many of the results reported on the digestibility of the nitrogen of these foods *in vivo* are in all probability only approximately correct, since they have been arrived at indirectly from results obtained with a mixture of nitrogen-containing foods.

The experimental data to be reported in this paper were obtained from a series of thirty rats fed rations in which ether-extracted cocoa powder supplied practically all of the nitrogen, or in which a definite mixture of skim milk powder and ether-extracted cocoa powder provided practically all of the nitrogen. The investigations consisted of nitrogen balance studies so designed as to obtain information on the biological value of the ab-

sorbed nitrogen as well as on the digestibility of the nitrogen as consumed.

Experimental Results.

The methods used in the metabolism experiments relative to the preparation of rations, the feeding of the rats, and the collection of the excreta have already been described in earlier reports (9) of similar experiments from this laboratory. In the initial and final standardizing periods, in which the relation of metabolic fecal nitrogen to the food consumed and of the endogenous nitrogen to the body weight were determined, the rations contained a small amount of dried ether-extracted whole egg to furnish from 0.70 to 0.75 per cent of nitrogen. In the test periods the nitrogen-containing constituents consisted of dried ether-extracted cocoa powder or of dried skim milk or of a mixture of the two in such a proportion that each food contributed 50 per cent of the nitrogen. In these test rations enough of the nitrogen-containing materials was included to furnish from 1.30 to 1.35 per cent of nitrogen (approximately 8 per cent of crude protein). The other constituents of the ration consisted of 4 per cent of the Osborne and Mendel salt mixture (10), 10 per cent of centrifuged butter fat, 4 per cent of Cellu Flour,[1] from 10 to 30 per cent of sucrose, and enough starch to make up 100 per cent.[2] The ingredients were mixed with water and cooked in a double boiler. After being dried and ground finely, the rations were analyzed for nitrogen.

Besides its allotment of the experimental rations each rat received daily 25 mg. of yeast vitamin (Harris) as a source of vitamin B and 1 to 2 drops of cod liver oil. The amount of vitamin B concentrate fed contained from 2 to 3 mg. of nitrogen, which has been neglected in the calculation of the coefficients of digestibility and of the biological value of the dietary nitrogen.

The cocoa used in these experiments was a common brand (Hershey's), which was found by analysis to consist of the following constituents: moisture 3.63, ether extract 24.63, ash 4.91,

[1] A product obtained from the Chicago Dietetic Supply House containing by actual analysis, 37.8 per cent crude fiber and only 0.015 per cent of nitrogen.

[2] In the later experiments 1 per cent of NaCl was included in the rations.

nitrogen 3.83, crude fiber 6.84 per cent, and gross energy 5.57 calories per gm. A theobromine determination by the Decker-Kunze method gave an exceptionally high value of 2.83 per cent. This cocoa powder was continuously extracted with ether for 48 hours or longer before being used in the experimental rations, in order to remove at least the larger part of the contained fat.[3] It has always been considered a wise plan in performing these metabolism studies to insure that the rations to be compared are approximately equal in calorific value.

The complete data of the nitrogen balance experiments with the resulting biological values are given in Table II.

The biological values calculated for these different rations presumably represent the percentage of the absorbed nitrogen used by growing rats for both maintenance and growth. Their calculation involves the assumption that the excretion of fecal nitrogen per gm. of food consumed on the low nitrogen ration in the first and final periods measures the excretion of body nitrogen in the feces in the intervening periods. Any change in the excretion of the fecal nitrogen per gm. of low nitrogen ration from the first to the final period is assumed to occur in a linear fashion with respect to time. The second assumption involved in these calculations is that the excretion of nitrogen in the urine per 100 gm. of body weight in the first and final periods of low nitrogen feeding is a measure of the excretion of body nitrogen in the urine in the intervening periods, any change in these values from the first to the final period being also assumed to be linear.

The reasoning upon which the calculation of the biological value is based may be illustrated by a concrete case; namely, that of Rat 334 in Period II. In 7 days comprising this collection period, this rat consumed an average of 5.6 gm. of food daily containing 73 mg. of nitrogen exclusive of that consumed in the vitamin B concentrate. The fecal nitrogen for the period averaged 56 mg. per day, of which it is estimated that 11 mg. were contributed by the body. Therefore, 45 mg. may be considered as representing indigestible food nitrogen. On this assumption, the absorbed nitrogen amounted to 73 − 45 = 28 mg. per day. The average daily excretion of urinary nitrogen was 34 mg. Since the endogenous metabolism of the rat was estimated to have resulted in the excretion of 13 mg. of nitrogen in the urine, the difference between this figure and the total urinary nitrogen, namely 21 mg., may be considered as resulting from the exogenous metabolism of the cocoa nitrogen. Since 28 mg. of the cocoa nitrogen were absorbed and 21 mg. were excreted in the urine, approximately 7 mg. may be considered as having been retained in the body for maintenance and growth. This amounts to 26 per cent of the

[3] Examination of the ether extract showed it to be practically free of nitrogen.

TABLE II.

Nitrogen Metabolism Data and Biological Values.

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
Period I. Egg ration, containing 0.74 per cent N.							
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
331	61	67	8.0		14	17	
332	59	65	8.0		14	17	
333	59	65	8.0		15	16	
334	59	63	8.0		15	18	
335	58	62	8.0		15	17	
Period II. Cocoa ration, containing 1.29 per cent N.							
331	52	48	5.4	69	54	32	22
332	51	47	5.2	67	53	29	34
333	51	49	5.1	66	49	30	33
334	53	49	5.6	73	56	34	26
335	50	48	5.7	74	54	33	35
Period III. Egg ration, containing 0.74 per cent N.							
331	55	56	5.7		12	11	
332	54	52	4.9		10	15	
333	57	58	5.8		13	11	
334	55	56	5.8		12	13	
335	56	56	5.6		11	14	
Period I. Egg ration, containing 0.74 per cent N.							
341	63	67	8.0		15	22	
342	60	63	7.9		15	20	
343	61	63	8.0		15	19	
344	62	64	8.0		14	20	
345	65	67	8.0		16	20	
Period II. Cocoa ration, containing 1.29 per cent N.							
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
341	56	50	5.8	75	58	33	41
342	52	48	6.8	88	59	28	66
343	54	48	5.7	74	40	31	65
344	53	44	5.6	72	60	28	47
345	55	50	5.9	76	63	30	38

TABLE II—*Continued.*

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
			Period III. Egg ration, containing 0.74 per cent N.				
341	56	63	6.8		13	17	
342	54	57	7.0		12	14	
342	55	56	5.7		14	14	
344	54	55	5.3		13	16	
345	57	61	6.1		16	12	
			Period I. Egg ration, containing 0.72 per cent N.				
361	70	85	8.0		22	18	
362	70	72	7.8		18	16	
363	89	94	8.0		20	20	
364	68	77	6.4		22	15	
365	81	87	8.0		20	20	
			Period II. Milk ration, containing 1.35 per cent N.				
361	92	100	8.0	108	30	38	82
362	79	89	6.8	92	24	34	80
363	98	106	8.0	108	29	36	84
364	86	95	7.0	95	24	29	87
365	90	100	8.0	108	30	34	87
			Period III. Cocoa-milk ration, containing 1.35 per cent N.				
361	98	97	8.0	108	52	43	68
362	86	85	7.3	100	61	41	55
363	106	108	8.0	108	57	42	68
364	93	95	7.9	107	62	38	68
365	99	98	7.9	107	61	41	68
			Period IV. Cocoa ration, containing 1.31 per cent N.				
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
361	91	80	5.4	71	57	31	44
362	72	68	4.6	60	46	32	17
363	101	94	6.2	81	70	34	27
364	88	78	4.3	56	48	28	24
365	93	82	4.8	63	66	30	

TABLE II—*Continued.*

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
Period V. Egg ration, containing 0.72 per cent N.							
361	86	81	3.6		11	12	
362	Died at end of Period IV*						
363	101	106	7.0		15	16	
364	86	81	3.8		12	11	
365	89	92	5.3		13	14	
Period I. Egg ration, containing 0.67 per cent N.							
391	119	115	6.5		17	24	
392	123	114	5.6		14	30	
393	115	112	6.4		16	24	
394	117	112	5.9		14	26	
395	115	108	5.6		15	26	
Period II. Cocoa-milk ration, containing 1.28 per cent N.							
391	114	119	8.0	103	53	36	77
392	112	115	8.0	103	54	36	78
393	110	113	8.0	103	51	38	73
394	110	111	8.0	103	55	36	74
395	107	110	8.0	103	51	38	75
Period III. Egg ration, containing 0.66 per cent N.							
391	125	125	5.4		11	18	
392	120	112	6.4		11	16	
393	118	119	6.1		12	16	
394	116	120	6.7		12	15	
395	115	113	4.7		10	15	

* In estimating the body N in the feces and urine of this rat in Periods II to IV, inclusive, it was assumed that the variations in the metabolic N in the feces per gm. of food consumed, and in the endogenous N in the urine per 100 gm. of body weight, were the same as the *average* variations for the other four rats in the group.

TABLE II—*Continued.*

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
			Period I. Egg ration, containing 0.67 per cent N.				
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
401	78	84	7.9		14	13	
402	77	79	6.8		16	13	
403	77	79	7.5		16	13	
404	75	79	7.5		17	12	
405	71	75	7.2		15	12	
			Period II. Cocoa-milk ration, containing 1.28 per cent N.				
401	84	89	7.5	96	50	27	75
402	82	87	8.0	103	58	30	71
403	81	85	7.9	102	54	29	75
404	79	80	6.8	87	50	25	75
405	78	77	7.9	102	57	30	70
			Period III. Egg ration, containing 0.66 per cent N.				
401	94	92	3.9		7.7	11	
402	91	91	3.1		6.4	11	
403	90	89	3.8		7.1	12	
404	85	83	3.2		7.8	11	
405	85	84	4.2		9.0	11	
			Period I. Egg ration, containing 0.67 per cent N.				
411	65	67	5.4		13	11	
412	61	63	5.9		15	10	
413	73	78	8.0		17	12	
414	61	62	5.7		12	10	
415	65	69	7.3		6.0	11	
			Period II. Cocoa-milk ration, containing 1.28 per cent N.				
411	65	70	5.4	70	37	23	76
412	64	67	5.1	66	40	22	71
413	75	83	7.3	93	55	29	67
414	62	67	5.3	68	41	22	69
415	68	73	6.1	79	46	24	66

TABLE II—*Concluded.*

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
Period III. Egg ration, containing 0.66 per cent N.							
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
411	69	66	2.7		6.1	12	
412	64	64	2.9		6.7	12	
413	81	81	4.5		9.2	11	
414	66	65	3.1		8.4	8.6	
415	74	72	3.3		6.3	9.8	

absorbed nitrogen and represents the biological value of the cocoa nitrogen in this particular experiment.

Table III contains a summary of the coefficients of digestibility, corrected for the metabolic nitrogen in the feces, and of the biological values of the nitrogen of cocoa in fifteen individual experiments. Considerable variation will be seen to exist within

TABLE III.

Digestibility and Biological Value of Nitrogen of Cocoa.

Rat No.	Digestibility.	Biological value.
331	38	22
332	35	34
333	41	33
334	38	26
335	41	35
341	37	41
342	46	66
343	63	65
344	33	47
345	35	38
361	42	44
362	40	17
363	30	27
364	38	24
365	12	
Average.....	38	37

these two groups of figures, which is probably the result of two conditions. The cocoa rations were not particularly palatable to the experimental rats, and as a result the intake of food was too small to maintain constant weight, even though the energy intake appeared to be sufficient. In most cases, the rats lost

TABLE IV.

Digestibility and Biological Value of Nitrogen of a Mixture of Milk and Cocoa Containing Equal Parts of the Nitrogen of Each.

Rat No.	Digestibility.	Biological value.
361	74	68
362	55	55
363	65	68
364	66	68
365	61	68
391	67	77
392	63	78
393	68	73
394	63	74
395	69	75
401	63	75
402	61	71
403	63	75
494	61	75
405	60	70
411	65	76
412	58	71
413	58	67
414	59	69
415	52	66
Average.....	63	70

weight continuously and appeared to be in a miserable condition at the end of the period. Concordant results cannot be expected with animals in a condition of marked malnutrition. In the second place, it is to be expected that the variation among individual determinations of digestibility and biological value will tend to increase as these values decrease. Given deviations

in the amount of nitrogen apparently digested, or in the amount of nitrogen apparently retained in the body from the food in individual experiments, due either to faulty technique, inaccurate assumptions, or inherent biological variation, will have the greater effect on the coefficients of digestibility and the biological values the smaller the amounts of absorbed nitrogen and retained nitrogen. The average coefficient of digestion of cocoa nitrogen in these fifteen tests is 38, while the average biological value of the digestible nitrogen is 37. These figures may perhaps be considered as fairly representative of cocoa when fed at a level of approximately 8 per cent of crude protein.

The protein value of a mixture of milk and cocoa containing the same amount of nitrogen from each was next investigated in metabolism experiments upon twenty rats. The results of this experiment relative to the coefficients of digestibility and the biological values are summarized in Table IV. This mixed ration was much more palatable to the rats than the ration of cocoa alone and was consumed in much larger amounts. Nevertheless it was evidently deficient in some essential constituent, since the amount of food consumed should have produced much larger gains in weight than were actually recorded. The results of the experiment are much less variable than those obtained with the cocoa alone and the averages may be considered as more significant. The average digestibility of this mixture of milk and cocoa nitrogen was 63 per cent and the average biological value, 70 per cent.

In one experiment on five rats the biological value of milk nitrogen alone was found to be 84. This value is practically identical with those previously reported (11). Assuming an average biological value for milk nitrogen and a digestibility of 100 per cent, it is possible to compute the digestibility and the biological value of a mixture of equal parts of milk and cocoa nitrogen, such as was fed in the second series of experiments, on the assumption than neither food influenced the utilization of the other. Taking the digestibility of cocoa nitrogen as 38 (see Table III), a mixture of equal parts of milk and cocoa nitrogen would be expected to have a digestibility of $\frac{100 + 38}{2} = 69$. This value is not far from that actually obtained for such a mix-

ture, namely 63 (see Table IV), and the approximation would be still closer if a digestibility of less than 100 is assumed for milk nitrogen. Assuming a digestibility of 95 would lower the estimated value to 66. These calculations, therefore, do not afford any basis for assuming any marked associative digestibility between these two foods.

TABLE V.

Daily Excretion of Creatine + Creatinine Nitrogen by Rats in Egg Ration Periods.

Rat No.	Initial period.				Final period.			
	Average body weight.	Creatine + creatinine N.			Average body weight.	Creatine + creatinine N.		
		Total per day.	Per kilo of body weight.	As per cent of urine N.		Total per day.	Per kilo of body weight.	As per cent of urine N.
	gm.	*mg.*	*mg.*		*gm.*	*mg.*	*mg.*	
331	63	1.31	21	7.6	56	1.36	24	12.5
332	61	1.30	21	7.5	53	1.32	25	8.9
333	61	1.28	21	7.9	57	1.30	23	12.0
334	62	1.28	21	7.2	55	1.30	24	10.1
335	59	1.28	22	7.5	55	1.34	24	9.6
341	64	1.45	23	6.7	59	1.47	25	8.7
342	61	1.39	23	7.0	55	1.42	26	10.2
343	62	1.48	24	7.6	55	1.49	27	10.6
344	63	1.58	25	7.8	54	1.52	28	9.7
345	65	1.44	22	7.3	58	1.47	25	12.5
361	78	1.34	17	7.4	84	1.25	15	10.1
362	70	1.31	19	8.2				
363	90	1.62	18	8.1	104	1.51	14	9.6
364	73	1.32	18	8.7	83	1.29	15	12.2
365	83	1.56	19	7.8	90	1.35	15	9.4

In predicting the biological value of a mixture of cocoa and milk nitrogen in the ratio of 1 to 1, the marked difference in digestibility must be taken into account. Although the ratio of total nitrogen consumed was as 1 to 1, the ratio of digested nitrogen would be quite different, due to the low digestibility of the cocoa nitrogen. Again, assuming 100 per cent digestibility for milk and 38 for cocoa, the ratio of digestible milk nitrogen to digestible cocoa nitrogen would be 2.6 to 1. Weighting the average

biological value (85) of milk nitrogen by 2.6 and that of cocoa nitrogen by 1, we obtain a biological value for the mixture of 72. This value is a remarkably close approximation to that actually obtained, namely 70, and again the approximation would be even closer if the digestibility of milk nitrogen were taken as less than 100. There is no reason to believe, therefore, that any supplementary relation exists between the digestible nitrogen of cocoa and that of milk. This finding is of considerable practical value in dietetics, since cocoa or chocolate is frequently consumed with milk either as a beverage or as a confection.

In most of the metabolism studies of this investigation the average daily excretion of total creatinine in the urine was determined[4] by a method described elsewhere (12). The results of this determination include the preformed creatinine as well as any creatine that might be present. A summary of the results for the egg ration periods, in which the nitrogen metabolism may be considered at the endogenous level, is given in Table V. The creatine coefficients of the rats in this experiment are quite similar to those previously reported from this laboratory and also to those computed from the data of Rose and Cook (13), relating to metabolism studies on twelve rats receiving a variety of diets, several of which were deficient in one or more indispensable amino acids. These creatinine coefficients, for rats weighing from 70 to 200 gm., ranged from 17 to 25 and averaged 21. Morgan and Osburn (14) have reported creatinine coefficients of 13 to 14 for growing and adult rats, while Folin and Morris (15) found an average value of 15 mg. of creatinine nitrogen per kilo of body weight per day for adult rats and 10 mg. for young rats.

An unexpected result of the creatinine studies was the marked increase in the excretion of total creatinine in the urine for rats on the cocoa rations. This increase is shown by the summaries contained in Tables VI and VII. There was an increase of approximately 80 per cent in the excretion of creatine + creatinine nitrogen in the cocoa periods over the egg ration periods. The cocoa-milk ration also induced a large increase in creatinine excretion in marked contrast to the milk ration alone. While the

[4] The creatinine determinations were made by Mr. R. L. Zimmerman.

creatinine excretion was increased slightly on the milk ration as compared with the egg ration, the increase was almost exactly in proportion to the increase in body weight. These findings suggest the occurrence of creatine or creatinine or both in commercial cocoa powder.

TABLE VI.

Effect of Ingestion of Cocoa on Daily Excretion of Creatine + Creatinine Nitrogen by Rats.

Rat No.	Egg ration.	Cocoa ration.	Egg ration.	Rat No.	Egg ration.	Cocoa ration.	Egg ration.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
331	1.31	2.35	1.36	341	1.45	2.51	1.47
332	1.30	2.40	1.32	342	1.39	2.58	1.42
333	1.28	2.33	1.30	343	1.48	2.58	1.49
334	1.28	2.28	1.30	344	1.58	2.47	1.52
335	1.28	2.09	1.34	345	1.44	2.60	1.47

TABLE VII.

Daily Excretion of Creatine + Creatinine Nitrogen on Rations Containing, Respectively, Egg, Milk, and a Mixture of Milk and Cocoa.

Rat No.	Egg ration.		Milk ration.		Milk-cocoa ration.		Egg ration.	
	Total excretion.	Excretion per kilo of body weight.	Total excretion.	Excretion per kilo of body weight.	Total excretion.	Excretion per kilo of body weight.	Total excretion.	Excretion per kilo of body weight.
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
361	1.34	17	1.58	16	2.64	27	1.25	15
362	1.31	19	1.50	18	2.91	34		
363	1.62	18	1.82	18	3.02	28	1.51	14
364	1.32	18	1.63	18	2.58	27	1.29	15
365	1.56	19	1.79	19	2.98	30	1.35	15

A qualitative examination of the original cocoa powder used in the metabolism experiments indicated the presence of a water-soluble compound or compounds giving the Jaffé color reaction. Furthermore, the intensity of the reaction was perceptibly increased by boiling the water extract with picric acid previous to the addition of alkali. Carrying out the reaction in a quantitative way and expressing the results as creatinine gave a percentage of

0.55 before boiling and 0.76 after boiling, both percentages referring to the total weight of cocoa used. An attempt definitely to isolate and identify creatine or creatinine in cocoa is now being made.

SUMMARY.

When fed to young growing rats at a level of approximately 8 per cent crude protein, the nitrogen of cocoa was found to possess an average coefficient of digestibility (corrected for metabolic nitrogen in the feces) of 38. The average biological value of the absorbed nitrogen of cocoa was found to be 37, though the variation among the fifteen individual determinations was considerable.

The average true digestibility of a mixture of milk and cocoa nitrogen in the ratio of 1 to 1, also fed at a level of approximately 8 per cent crude protein, was found to be 63. Assuming the true digestibility of milk nitrogen to be 100 and that of cocoa nitrogen to be 38, the expected digestibility of a mixture of the two in equal amounts would be 69. If the digestibility of milk nitrogen is taken as 95 instead of 100, the estimate is reduced to 66.

An average biological value for milk nitrogen of 84 was obtained in metabolism studies on five rats. This is almost identical with the values previously reported.

For a mixture of milk and cocoa nitrogen in a proportion of 1 to 1, an average biological value of 70 was obtained. Assuming an average biological value of 85 for milk nitrogen, and making allowance for the great difference in the digestibility of milk and cocoa nitrogen, the estimated biological value of the cocoa nitrogen is 31. This indirect estimate of the biological value of cocoa nitrogen checks fairly well with that actually determined, *i.e.* 37, and the agreement would be closer if the digestibility of milk nitrogen were assumed to be less than 100. If a digestibility of 95 is assumed, the estimate for the biological value of cocoa nitrogen is raised to 40.

Such calculations may be considered both as a confirmation of the determined biological value for cocoa nitrogen and as a strong indication that no marked supplementary relation exists between the nitrogenous compounds of milk and of cocoa, since, if such a

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relation existed, the estimated value for cocoa should be distinctly higher than the determined value.

Cocoa, with an average crude protein content of 21.5 per cent, would, according to these investigations, contain 8.2 per cent of digestible crude protein, and only 3.0 per cent of "net" protein, available for replenishing and enlarging the protein content of the animal body. Evidently cocoa must be classed as an unimportant protein food. The same conclusion applies with even greater force to chocolate, which contains a smaller percentage of crude protein than cocoa.

The ingestion of cocoa markedly increased the excretion of creatine + creatinine by rats, as determined by the reaction of Jaffé. An examination of the cocoa by this reaction indicated the presence in it of 0.55 per cent of creatinine and 0.24 per cent of creatine.

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THE INFLUENCE OF HYDRAZINE AND ITS DERIVATIVES ON METABOLISM.

II. CHANGES IN THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF THE BLOOD AND IN THE METABOLISM OF INJECTED GLYCINE IN HYDRAZINE INTOXICATION.*

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INTRODUCTION.

Mann and his coworkers (1–4) have recently reviewed the rôle of the liver in many important metabolic processes and, with the aid of the improved operative technique for hepatectomy developed by him, have brought forward new and convincing evidence of the function of the liver in the regulation of blood sugar content, in the deamination of amino acids, in the formation of urea, and in the destruction of uric acid.

There is another method available by which these hepatic functions may be demonstrated; namely, by the use of chemical substances which have an effect primarily upon the liver cells without producing marked injury to other tissues. Among these substances is hydrazine, which has been shown by Wells (5) to act specifically upon the parenchymatous cells of the liver. Although many investigations have been concerned with the influence of hydrazine on carbohydrate metabolism, little attention has apparently been given to the question of protein or uric acid metabolism in the hydrazinized animal. It is the purpose of the present paper to present data obtained on the influence of

* The material presented in this and the following paper comprises an abstract of a part of the thesis presented by S. Izume in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of the University of Michigan.

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hydrazine poisoning in the rabbit upon the amino acid, urea, and uric acid content of the blood, and upon the glyconeogenesis following ingestion of a simple amino acid, glycine.

It has been observed by Underhill (6), MacAdam (7), and Bodansky (8) that the subcutaneous injection of hydrazine sulfate produces a notable hypoglycemia. Underhill (9) has also reported that the total solids of the blood are increased in hydrazine poisoning, an increase associated with the anhydremia produced (10, 11), which has been ascribed to the fluid loss through vomiting. Underhill and Baumann (12) have demonstrated a hyperlipemia in hydrazinized dogs, the maximum content of blood fat being definitely related to the hypoglycemia. Hendrix and McAmis (13) observed an alkalosis in hydrazine poisoning, but were unable to show any causal relation between the alkalosis and the hypoglycemia. The ratio of sodium to chlorine in the serum was found to be increased in hydrazine intoxication. Bodansky (14) has reported one experiment in which he noted a marked increase in the non-protein nitrogen of the blood of a dog poisoned with hydrazine. Underhill and Kleiner (15) found that the urinary nitrogen and sulfur were little altered in dogs after the administration of hydrazine, but the content of creatinine and phosphorus was somewhat increased. No lactic, hydroxybutyric, or diacetic acid, no acetone and no glucose or other reducing substances were present in the urine. MacAdam (7) observed a creatinuria in hydrazine intoxication, which was closely associated with the hypoglycemia. These results were confirmed by Underhill and Baumann (16) who also noted a decreased hydrogen ion concentration of the urine.

In addition to the histological demonstration of liver injury in hydrazine poisoning (5), functional tests have also indicated impairment of the activity of the hepatic cells. The rate of excretion of injected phenoltetrachlorophthalein (17) is greatly decreased in dogs after hydrazine administration. The ability to synthesize hippuric acid after the administration of sodium benzoate is diminished (18). The tolerance for fructose is also lessened (14). Fiske and Karsner (19), however, in perfusion experiments, were not able to demonstrate any difference between normal livers and livers poisoned with hydrazine in their ability to lower the ammonia content of the perfusion fluid.

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EXPERIMENTAL.

Methods.

Rabbits which had fasted for several days were used in this investigation. Blood was usually drawn from the marginal ear vein, but occasionally from the heart, sodium citrate being used as anticoagulant. Both hydrazine sulfate and glycine were dissolved in physiological salt solution and injected subcutaneously. The animals received no food during the course of the experiments, but were given 50 to 75 cc. of water twice daily through a stomach tube. It was hoped by this means to avoid any marked concentration of the blood, which might be associated with the fasting and hydrazine intoxication.

For the determination of sugar, non-protein nitrogen, urea nitrogen, and amino nitrogen of the blood, and amino nitrogen of the urine, the methods of Folin and Folin and Wu were used. Uric acid in the blood was estimated directly in the Folin-Wu tungstic acid filtrate by Benedict's method. The glycogen content of the liver was determined by Pflüger's method with a slight modification in the technique of the final determination of reducing sugar. After the conversion of glycogen into glucose in the usual way, the solution was decolorized with Lloyd's reagent and the glucose was then determined by the Folin-Wu method.

Results.

Influence of Hydrazine on Amino Acid, Urea, and Uric Acid Content of Blood.

Our experiments judged both from the chemical findings and from the results of pathological examination of the liver and kidneys fall into two rather clearly defined groups. Microscopic examination[1] of the tissues by Dr. A. S. Warthin of the Department of Pathology showed that a sufficient dose of hydrazine sulfate (130 mg. per kilo) when administered subcutaneously to rabbits always produced to some extent a fatty degenerative infiltration of the liver. This finding was constant in practically all of our animals. In about half of these, however, the kidneys

[1] The pathological examination and diagnosis were made by Dr. A. S. Warthin, to whom we wish to take this opportunity to express our thanks for his hearty cooperation.

were also affected, inflammation and fatty degenerative infiltration being observed.

In those animals in which kidney involvement resulted following

TABLE I.

Rabbit 109, female, weight 1.9 kilos.

Date.	Hydrazine sulfate injected.	Blood content of:					Remarks.
		Sugar.	Non-protein nitrogen.	Urea nitrogen.	Amino acid nitrogen.	Total color with uric acid reagent calculated as uric acid.	
1926	*mg. per kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
June 8							6 days fasting.
9.00 a.m.		109	60.7	26.5	10.3	1.80	
10.50 "	130						
4.50 p.m.		163	66.7	30.1	16.7	2.23	
10.50 "		122	74.5	37.0	18.4		
June 9							
10.50 a.m.		110	105.3	55.2	17.6		
10.50 p.m.		150	148.2	84.8	17.5	2.76	Slight depression.
June 10							
10.50 a.m.		125	165.8	114.3	20.3	2.89	
11.30 "	130						
1.30 p.m.		74	210.0	124.0	30.4	4.36	Marked depression.
2.00 "							Death.

Pathological Report.—Kidney showed very marked lesions. Throughout the cortex there were areas of atrophy and fibrosis with some inflammatory infiltration involving a whole labyrinth. The tubules were in part atrophic and in part dilated with atrophy of the cells, but the glomeruli were relatively well preserved in these areas. In some of the dilated tubules a finely granular bluish-staining precipitate was present. Some of the tubules showed a slight lipoidosis of renal cells. The inflammatory process extended clear through from the cortex to the portion of the papilla belonging to the part involved.

Liver: slight fatty degenerative infiltration. Atrophy of large bile ducts. Slight lymphocyte infiltration of some of the periportal islands.

the injection of hydrazine, a marked rise in the non-protein nitrogen and in the urea nitrogen occurred (Table I). It was noted that, whenever the blood showed a marked retention of

urea nitrogen, the kidney injury was also present. We consider that the rise in the urea content of the blood is associated with a disturbance of kidney function and that the hepatic injury is not necessarily involved.

TABLE II.

Rabbit 113, female, weight 2.4 kilos.

Date.	Hydrazine sulfate injected.	Blood content of:					Remarks.
		Sugar.	Non-protein nitrogen.	Urea nitrogen.	Amino acid nitrogen.	Total color with uric acid reagent calculated as uric acid.	
1926	*mg. per kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
July 2							7 days fasting.
9.40 a.m.		95	60.6	32.5	10.3	2.29	
10.15 "	130						
1.15 p.m.		87	62.0	33.3	12.1	2.86	
4.15 "		62	61.7	25.8	15.8	2.47	
7.15 "		105	72.3	26.0	22.4	2.38	
10.15 "		154	84.5	30.2	26.0	2.38	
July 3							
10.15 a.m.		111	102.5	32.8	32.2	2.96	Depression, blood from heart.
July 4							
10.15 a.m.		111	86.9	33.8	31.1	3.86	Still in depression.
July 5							
10.15 p.m.		106	70.2	33.6	13.5	2.96	Apparently normal. Killed.

Pathological Report.—Postmortem microscopical examination showed: (1) Liver: Moderate passive congestion. Slight small celled infiltration of many islands. Slight hepatitis. Slight atrophy of liver cells without fatty infiltration but scattered cells showed large fat droplets (slight fatty infiltration). No necrosis. (2) Kidney: Very slight cloudy swelling, possibly post mortem. No other pathology.

In the absence of kidney lesions, the content of non-protein nitrogen, amino acid nitrogen, and uric acid always increased prominently and progressively following the injection of hydrazine sulfate, while the urea nitrogen remained almost constant (Tables II and III). Of the changes in the composition of the blood, the

increase in the amino acid nitrogen was most significant, its maximum content being two to four times the normal value for the fasting rabbit. The increase cannot be considered to be due to any concentration of the blood, since the urea nitrogen was practically unchanged and the sugar content frequently much lowered. No definite relationship between the degree of hypoglycemia and the increase of amino acid nitrogen was noted, but

TABLE III.

Rabbit 106, female, weight 2.3 kilos.

Date.	Hydrazine sulfate injected.	Blood content of:					Remarks.
		Sugar.	Non-protein nitrogen.	Urea nitrogen.	Amino acid nitrogen.	Total color with uric acid reagent calculated as uric acid.	
1926	*mg. per kg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
June 2							6 days fasting.
4.20 p.m.		107	51.5	24.5	14.1	1.27	
5.15 "	100						
June 3							
12.15 a.m.		132	55.3	24.2	19.5	1.31	
8.15 "		142	61.4	25.8	24.7	1.50	
10.05 "	100						
4.05 p.m.		42	72.1	24.7	31.8	3.66	Coma.
6.05 "		39	78.4	23.5	34.3	3.70	Condition continued.
7.00 "							Death.

Pathological Report.—(1) Liver: Very slight fatty degenerative infiltration. Congestion. (2) Kidney: Congestion. Slight cloudy swelling. No other changes found.

it was observed that the progressive increase in the amino acid nitrogen was usually paralleled by the increase in uric acid.[2] The increased amino acid content was most marked when the animals

[2] We use the term "uric acid" content with the realization that the substance determined by Benedict's direct method, particularly in rabbit blood, may consist in large part of other substances which react with the arsenophosphotungstic acid reagent.

showed severe intoxication. Thus Rabbit 113 developed the maximum increase (213 per cent of the normal content) 24 hours after the administration of hydrazine when the animal showed severe symptoms of intoxication. During the intoxication, the blood amino acid content continued high, but when the animal recovered from the symptoms and appeared normal 84 hours after the injection, the amino acid content was also found to have returned nearly to the normal value. It should be noted that the greater part of the increase in the non-protein nitrogen is accounted for by the rise in the amino acid nitrogen. The results of these and other similar experiments suggest that in hydrazine

TABLE IV.

Effect of Subcutaneous Injection of Glycine upon Amino Acid Nitrogen Content of Blood of Normal Fasting Rabbits.

Rabbit No.	Fasting.	Glycine injected per kilo.	Amino acid nitrogen in 100 cc. blood.					
			Initial.	1 hr.	3 hrs.	6 hrs.	9 hrs.	12 hrs.
	days	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
99*a*	2	1.0	8.23	17.5	17.5	14.0		
101*a*	2	1.0	8.14	18.5	17.5	14.3		
102	5	1.0	9.95		26.2	21.4	13.4	
103	5	1.0	8.58		22.5	18.4	12.3	
108	6	1.0	8.91	24.2	23.1	16.8	10.3	9.5
99*b*	4	3.0*	8.23	49.0	47.2	47.0		40.0
101*b*	4	3.0*	8.14	46.4	48.2	45.3		43.8

* Previous injection of glycine (1 gm. per kilo) on the 2nd day of fasting.

poisoning, the injury to the liver results in a disturbance of protein metabolism as evidenced by the failure of deamination of amino acids and their accumulation in the blood.

Influence of Hydrazine on Amino Acid Tolerance of Rabbits and on Glyconeogenesis Following Administration of Glycine.

Further evidence of the failure of the animal organism to metabolize the protein derivatives satisfactorily in hydrazine poisoning was afforded by comparative studies of the behavior of glycine injected subcutaneously into normal and hydrazinized rabbits. As shown in Table IV, when glycine in moderate amounts (1 gm. per kilo) was injected into rabbits previously fasted for short periods,

the amino acid nitrogen content of the blood increased to two to three times the initial value within an hour, and remained high for several hours, gradually returning to the normal content within 9 to 12 hours. With larger amounts of glycine (3 gm. per kilo) the increase was much greater and the high level of blood amino acid nitrogen persisted for more than 12 hours.

When glycine (1 gm. per kilo) was injected after previous injec-

TABLE V.

Effect of Subcutaneous Injection of Glycine upon Amino Acid Nitrogen Content of Blood under Influence of Hydrazine Poisoning.

Rabbit No.	Fasting.	Administration of:		Amino acid nitrogen in 100 cc. blood.								Remarks.
		Hydrazine sulfate per kilo.	Glycine per kilo.	Initial.	After hydrazine.		After glycine.					
					3 hrs.	6 hrs.	1 hr.	3 hrs.	6 hrs.	9 hrs.	12 hrs.	
	days	*mg.*	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	
102	6	130	1.0	9.7	12.4	17.5		37.0	40.3	42.0		Glycine given 7 hrs. after hydrazine.
103	6	130	1.0	11.0	12.9	14.9		38.5	47.3	52.5		Glycine given 6.5 hrs. after hydrazine.
108	7	130	1.0	8.5	12.5	15.5	35.6	40.2	46.0	49.2	49.0	Glycine given 7 hrs. after hydrazine.
99	6	130	3.0	8.2	15.5*		63.7	86.5	95.5			Glycine given 5 hrs. after hydrazine.
101	6	130	3.0	8.1	16.6*		73.3	82.5	86.5			" "

* Sample was collected 4 hours after the injection of hydrazine.

tion of hydrazine (Table V), the amino acid content of the blood, which was already somewhat increased as a result of the hydrazine injection, increased to a maximum of three to four times the initial value, and remained at this high level for more than 12 hours. With larger amounts of glycine (3 gm. per kilo), the blood amino acid had increased tenfold in 6 hours, when the animals were killed for the determination of glycogen in the liver.

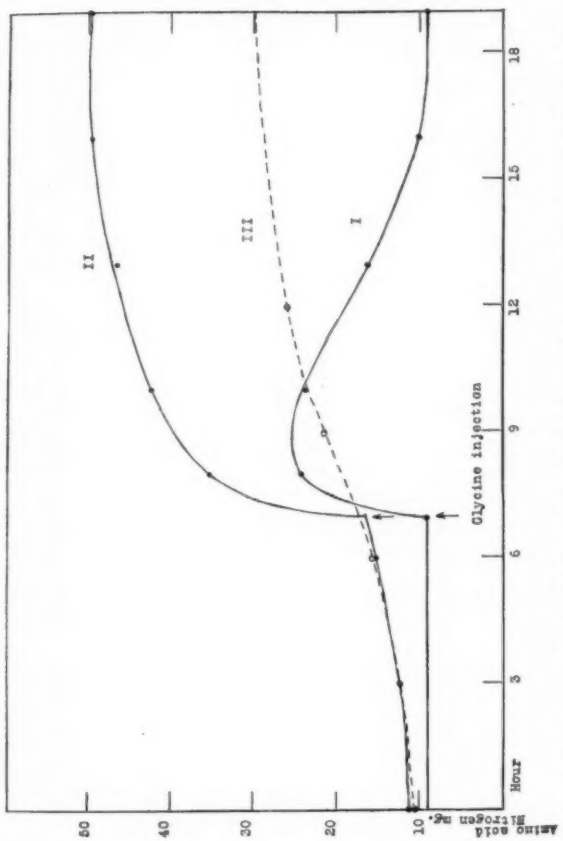

FIG. 1. A comparison of the amino acid nitrogen content of the blood following the injection of 1 gm. of glycine per kilo (*a*) in a fasting rabbit (No. 108) (Curve I) and (*b*) in the same rabbit after hydrazine poisoning (Curve II). Curve III represents the changes in amino acid nitrogen of the blood in a fasting rabbit (No. 113) which had been injected with hydrazine but had received no glycine.

A graphic representation of this difference between normal and hydrazinized animals is given in Fig. 1, in which are shown the curves for the amino acid nitrogen of the blood following the

TABLE VI.

Effect of Subcutaneous Injection of Glycine upon Urea Nitrogen Content of Blood in Inanition and after Administration of Hydrazine.

Rabbit 108, male, weight 1.9 kilos.

Date.	Hydrazine sulfate injected.	Glycine injected.	Blood content of:		
			Sugar.	Amino acid nitrogen.	Urea nitrogen.
1928	*mg. per kg.*	*gm. per kg.*	*mg.*	*mg.*	*mg.*
June 1*					
8.45 a.m.			109	8.9	26.2
8.55 "		1			
9.55 "			125	24.2	27.4
11.55 "			111	23.1	20.7
2.55 p.m.			105	16.8	34.5
5.55 "			100	10.3	32.2
8.55 "			111	9.5	30.3
June 2					
8.10 a.m.			102	8.5	27.6
8.50 "	130				
11.50 "			75	12.5	26.1
2.50 p.m.			56	15.5	23.4
3.20 "		1			
4.20 "			98	35.6	23.2
6.20 "			95	40.2	25.5
9.20 "			82	46.0	25.0
June 3					
12.20 a.m.			126	49.2	27.1
3.20 "			135	49.0	25.1
11.50 "			100	32.0	26.8
11.50 p.m.			105	31.5	26.5
June 5					
10.00 a.m.			112	9.8	27.5

* The animal fasted for 6 days previous to the beginning of the experiment.

injection of 1 gm. of glycine per kilo in the fasting rabbit (Curve I), and in the same animal under the influence of hydrazine intoxication (Curve II). For comparison, the changes in the blood amino acid content of another rabbit poisoned with hydrazine, but to

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which no glycine was administered, are given (Curve III). It is evident that the ability of the organism to metabolize injected glycine is greatly impaired in hydrazine poisoning.

In the normal fasting rabbit the urea content of the blood after the injection of glycine (1 gm. per kilo) increased gradually and reached its maximum value about 6 hours after the injection. In the hydrazinized rabbit, on the other hand, even though the amino acid nitrogen of the blood increased greatly above the normal after the injection of glycine, no rise in the urea nitrogen was observed. A typical protocol is presented in Table VI in which are given the values for blood urea of Rabbit 108, the same animal for which

TABLE VII.

Effect of Subcutaneous Injection of Glycine upon Sugar Content of Blood.

Rabbit No.	Fasting.	Glycine injected per kilo.	Blood sugar content in 100 cc. blood.					
			Initial.	1 hr.	3 hrs.	6 hrs.	9 hrs.	12 hrs.
	days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
99*a*	2	1.0	0.118	0.132	0.117	0.112		
101*a*	2	1.0	0.116	0.120	0.111	0.107		
102	5	1.0	0.124		0.129	0.121	0.105	
103	5	1.0	0.134		0.123	0.116	0.111	
108	6	1.0	0.109	0.125	0.111	0.105	0.100	0.111
100*a*	2	1.0	0.124	0.131	0.128	0.118		
99*b*	4	3.0*	0.118	0.154	0.155	0.148		0.143
101*b*	4	3.0*	0.116	0.143	0.143	0.143		0.141
100*b*	4	3.0*	0.124	0.150	0.141	0.133		0.123

* Previous injection of glycine (1 gm. per kilo) on the 2nd day.

curves of blood amino acid content are plotted in Fig. 1. The constancy of the values for urea nitrogen obtained in this experiment would indicate that the high values for amino acid nitrogen cannot be due to concentration of the blood as a result of the hydrazine intoxication.

The blood sugar of fasting rabbits was little affected by the injection of glycine in doses of 1 gm. per kilo (Table VII). In most animals the sugar content of the blood remained unaffected, but occasionally (*e.g.* Rabbits 99*a* and 108, Table VII) a slight transient hyperglycemia was observed 1 hour after the administration of glycine. However, when larger doses of glycine (3

gm. per kilo) were injected, the blood sugar content increased about 20 to 30 per cent in an hour and remained high for several hours. In the hydrazinized animals, even when 3 gm. of glycine per kilo were injected, no significant change in the blood sugar was

TABLE VIII.

Effect of Subcutaneous Injection of Glycine upon Sugar Content of Blood under Influence of Hydrazine Poisoning.

Rabbit No.	Fasting.	Administration of:		Sugar content of 100 cc. blood.								Remarks.
		Hydrazine sulfate per kilo.	Glycine per kilo.	Initial.	After hydrazine.			After glycine.				
					3 hrs.	4 hrs.	6 hrs.	1 hr.	3 hrs.	6 hrs.	9 hrs.	
	days	*mg.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	
102	6	130	1.0	0.133	0.145		0.132		0.138	0.133	0.158	Glycine given 7 hrs. after hydrazine.
103	6	130	1.0	0.134	0.159		0.131		0.111	0.108	0.100	Glycine given 6.5 hrs. after hydrazine.
108	7	130	1.0	0.102	0.075		0.056	0.098	0.095	0.082	0.126	Glycine given 7 hrs. after hydrazine.
99	6	130	3.0	0.118		0.153		0.146	0.143	0.160		Glycine given 5 hrs. after hydrazine.
101	6	130	3.0	0.116		0.111		0.116	0.120	0.166		" "

apparent (Table VIII). Thus the blood sugar of Rabbit 99 which had increased from 0.118 to 0.154 per cent 1 hour after the glycine injection (Table VII) showed no change after glycine when the animal had been previously poisoned with hydrazine (Table

VIII). It is evident that the transformation of glycine to glucose does not proceed normally under the influence of hydrazine intoxication.

TABLE IX.

Glycogen Content of Liver Following Subcutaneous Injection of Glycine to Normal and Hydrazinized Rabbits.

Rabbit No.	Body weight.	Previous period of fasting.	Injection.		Glycogen content of liver.	Remarks.
			Hydrazine sulfate.	Glycine.		
	kg.	*days*	*mg. per kg.*	*gm. per kg.*	*per cent*	
111	1.6	7	0	0	0.0927	Control animal.
8	1.2	4	100	0	0.136	Died 1 hr. after hydrazine injection.
109	1.9	8	130 130	0	0.047	Died 2.5 hrs. after last dose of hydrazine. Hydrazine given on 6th and 8th days.
7	2.8	7	100 100 100	0	0.080	Died 4 hrs. after last dose of hydrazine. Hydrazine given on 4th, 6th, and 7th days.
106	2.3	7	100 100	0	0.058	Died 6 hrs. after last dose of hydrazine. Hydrazine given on 6th and 7th days.
102	2.0	6	130	1.0	0.147	Killed 9 hrs. after glycine injection. Hydrazine given 7 hrs. before glycine.
103	2.3	6	130	1.0	0.087	" "
104	2.4	7	0	3.0	0.061	Killed 9 hrs. after glycine injection.
105	2.9	6	0	3.0	0.624	" "
112	1.7	7	0	3.0	0.762	" "
99	2.6	6	130	3.0	0.218	Killed 7 hrs. after glycine injection. Hydrazine given 5 hrs. before glycine.
101	2.0	6	130	3.0	0.126	" "

A study of the deposition of glycogen after glycine injection was made in hydrazine poisoning (Table IX). In accord with the work of previous investigators we have observed that very small quantities of glycogen are present after fasting and after fasting

and hydrazine intoxication (Rabbits 7, 8, 106, 109, 111). The contents of glycogen in the liver of two rabbits (Rabbits 99 and 101), which after 6 days fasting were injected with hydrazine sulfate and 5 hours later with glycine (3 gm. per kilo), were found to be 0.218 and 0.126 per cent. Control rabbits similarly treated but which received no hydrazine (Rabbits 104, 105, and 112) stored 0.061, 0.624, and 0.762 per cent glycogen in the liver, indicating in two of the three animals the formation of appreciable amounts of glycogen after glycine injection. It appears that both glucose and glycogen formation from glycine are inhibited as a result of the injury to the liver produced by hydrazine.

DISCUSSION.

The marked increase in the amino acid content of the blood following the administration of hydrazine, might be considered to be due to an increased catabolism of tissue proteins, to a decreased ability of the organism to deaminize and utilize amino acids, or to a difficulty in excreting amino acids through the kidneys. Underhill and Kleiner (15) observed that the excretion of total nitrogen and sulfur in animals during hydrazine poisoning was only slightly different from that obtained during a comparable period of inanition. This fact seems to indicate that hydrazine probably does not induce an increased catabolism of tissue proteins to any considerable extent. The fact, which has been demonstrated by the authors, that the increase in amino acids in the blood in hydrazine poisoning was not always accompanied by an increase in blood urea, indicates that the accumulation of amino acids in the blood cannot always be ascribed to the renal injury which has been shown to be produced sometimes in rabbits by hydrazine.

It would seem reasonable, therefore, to attribute the increase of the amino acid content of the blood to injury of the liver which is known to occur in hydrazine poisoning, through which the power to deaminize and metabolize amino acids is disturbed. There is considerable evidence that the liver is an organ importantly concerned with the deamination of amino acids and the formation of urea. Severe injuries to or extirpation of the liver might be expected to cause an accumulation of amino acids in the blood and a decrease in the urea content of the blood and urine. Bollman, Mann, and Magath (3) studied the effect of complete hepatectomy

on the formation of urea in dogs and noted that the amino acid content of the blood increased progressively while the blood urea decreased considerably. A high amino acid content of the blood has also been observed in hepatic disease, acute yellow atrophy, cirrhosis, tumor, etc. (20–24). These facts lead us to believe that the increased amino acid content of the blood after the injection of hydrazine are due to the derangement of hepatic function produced by the poison.

The changes of amino acid and urea content of the blood observed in fasting rabbits after the injection of glycine in our experiments are in accord with those obtained by Van Slyke and Meyer (25) and Seth and Luck (26). They are also confirmed by unpublished experiments of Johnston in this laboratory. Van Slyke and Meyer also found that after the intravenous injection of 0.853 gm. of glycine per kilo into a dog, only 3.5 per cent of the injected glycine was recovered in the urine. In our fasting rabbits, the increases in the amino acid nitrogen in the urine after the subcutaneous administration of 1 gm. of glycine per kilo are comparable to that of Van Slyke, 1.2 to 6.7 per cent of injected glycine being excreted in the urine.

The decreased ability of the hydrazinized rabbit to deaminize and metabolize injected glycine, which we attribute to liver injury, is also in harmony with other experimental evidence. Rosenbaum (21) introduced a mixture of amino acids, "rectamin," in doses equivalent to 40 to 60 mg. of amino nitrogen per kilo subcutaneously into normal individuals and into patients with hepatic diseases (tumor, cirrhosis, yellow atrophy, etc.). In normal individuals the change in the amino acid nitrogen content of the blood was not significant, while in cases of hepatic disease a marked hyper amino acidemia was always observed within a short time after the injection. Similar results are reported by von Falkenhausen (24), Glaessner (27), Masuda (28), and Bier (29).

SUMMARY.

1. Subcutaneous administration of hydrazine sulfate to fasting rabbits often produced injury to the kidneys, as well as fatty degeneration of the liver.

2. Hydrazine always caused a marked rise in the amino acid content of the blood. The urea content of the blood following the

injection of hydrazine to rabbits was not increased when the kidneys were not affected by the hydrazine, but often increased markedly when the kidneys showed lesions.

3. In hydrazine poisoning the power of the animal organism to metabolize injected glycine, to transform glycine into glucose and urea, and to synthesize glycogen from glycine, was diminished.

4. The results are ascribed to the derangement of the function of the liver which results in the failure of normal deamination of amino acids. These results also furnish evidence that the liver is importantly concerned with deamination of amino acids and formation of urea.

Addendum.—After this paper was submitted for publication, further evidences of the similarity between the results observed in hydrazine poisoning and total hepatectomy were reported by Bollman, Mann, and Magath (30). In hepatectomized dogs it was demonstrated that no deaminization of injected amino acids occurred, and no formation of urea could be observed. It was also seen that no glucose was formed from amino acids (glycine, alanine) after the removal of the liver. Results obtained in dogs after hepatectomy by these observers are quite comparable to the results we have obtained after amino acid injection in hydrazinized rabbits.

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THE INFLUENCE OF HYDRAZINE AND ITS DERIVATIVES ON METABOLISM.

III. THE MECHANISM OF HYDRAZINE HYPOGLYCEMIA.

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INTRODUCTION.

The mechanism of the hypoglycemia produced by the injection of hydrazine sulfate has been studied from various points of view by Underhill and his coworkers. They finally concluded from their studies on the respiratory metabolism that the hypoglycemia was probably due to an increased combustion of carbohydrate. Without enumerating the facts which indicate that the hypoglycemia and other physiological effects produced by hydrazine are quite different from those produced by the internal secretion of the pancreas, insulin, it is evident that the effect of hydrazine on carbohydrate metabolism cannot be explained satisfactorily by the hypothesis of Underhill.

In a previous paper of this series (1) the authors have reported that the administration of hydrazine to fasting rabbits caused the derangement of liver function, which was manifested in an increase in the amino acid content of the blood and in a decrease in the ability to metabolize injected glycine, to transform glycine to glucose and urea, and to synthesize glycogen in the liver from glycine. In the present paper, experimental evidence is presented which indicates that the hypoglycemia following the injection of hydrazine is also closely associated with hepatic injury, which results in the failure of glyconeogenesis in the transformation of amino acids to glucose. On basis of these observations a theory of the mechanism of hydrazine hypoglycemia is suggested which differs somewhat from that of Underhill.

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Since Underhill's discovery of the production of hypoglycemia by hydrazine (2), the mechanism of the hypoglycemia has been studied by various methods in his laboratory. Underhill and Fine (3) were able to prevent hyperglycemia and glycosuria in depancreatized dogs by the injection of hydrazine, and were able to demonstrate the presence of epinephrine in the adrenals of hydrazinized dogs. These results led them to the conclusion that hydrazine probably induced hypoglycemia by increasing the efficiency of the pancreatic secretion or by augmenting its output, since no inhibitory influence on the adrenal secretion was evidenced. The glycogen content of the liver and muscles of dogs was greatly decreased following the administration of hydrazine (4). Underhill and Prince (5) were unable to demonstrate any differences in the degree of utilization of glucose in the perfusion of the surviving hearts of normal and hydrazinized rabbits. Rabbits poisoned with hydrazine showed a marked retardation in the utilization of glucose introduced subcutaneously (6). The glyoxalase activity of the liver was not altered by hydrazine (7). Underhill and Murlin (8) observed that the administration of hydrazine to fasting dogs caused an increased respiratory quotient. The heat production of these animals was not increased, however, to any extent, by the hydrazine. They concluded that an increased combustion of carbohydrate, evidenced by the greater respiratory quotient, was the probable cause of the hypoglycemia.

If, in hydrazine intoxication, there is sufficiently great increase in carbohydrate combustion to account for the hypoglycemia observed, then it would be anticipated that the glucose administered would be more rapidly utilized by the animal under the influence of hydrazine than by the normal animal. Underhill and Hogan (6), however, were unable to show that hydrazine led to a more rapid utilization of glucose by the organism, but, on the contrary, found that the hyperglycemia following the subcutaneous injection of glucose persisted longer in hydrazinized animals than in normal animals. The authors have repeated the experiments of Underhill and Hogan and were not only able to confirm their results, but have offered further evidence which indicates that this decrease in the tolerance for glucose is to be

ascribed to an impairment of the glycogenetic function of the liver produced by hydrazine.

Underhill has noted a difference in response to injected glucose between dogs and rabbits in hydrazine intoxication. Hydrazinized dogs were killed by the subcutaneous injection of 5 gm. of glucose per kilo, while rabbits under the same conditions showed marked and persistent hyperglycemia, but survived the injections. Bodansky (9) who studied the glucose tolerance of dogs in hydrazine poisoning does not appear to have observed the death of his animals after glucose injection as did Underhill.

EXPERIMENTAL.

In these experiments, slightly larger doses of hydrazine sulfate and smaller amounts of glucose than those employed by the former investigators were used in order to produce more effective hydrazine intoxication and to prevent, as far as possible, the excretion of glucose into the urine. The rabbits received no food throughout the course of the experiments, but 50 to 75 cc. of water were given through a stomach tube twice daily. After a preliminary period of fasting, usually of 2 days duration, rabbits were injected subcutaneously with hydrazine sulfate in doses of 130 mg. per kilo. Several hours after, when the animals usually showed abnormal symptoms, 1.85 gm. of glucose (in 25 per cent solution) per kilo were injected subcutaneously. The sugar content of the blood was determined by the Folin-Wu procedure as indicated in the tables. 6 to 8 hours after the injection of glucose the animals were killed and the glycogen content of the liver was determined by a slight modification of Pflüger's method (1). The control animals received no hydrazine but were treated similarly in other respects.

The data presented in Table I indicate that, after the injection of glucose (1.85 gm. per kilo) to fasting rabbits, the concentration of blood sugar was increased by about 50 per cent at its maximum and usually returned to the normal level within 3 hours. They also demonstrate a considerable storage of glycogen in the liver 6 hours after the administration of glucose.

When, however, the same dosage of glucose was injected into fasting rabbits which had been previously poisoned with hydra-

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TABLE I.

Glucose Tolerance of Normal Rabbits.

Rabbit No.	Weight.	Fasting.	Injection of glucose per kilo.	Blood sugar content.							Liver glycogen.
				Initial.	After injection of glucose.						
					0.5 hr.	1 hr.	1.5 hrs.	2 hrs.	3 hrs.	6 hrs.	
	kg.	*days*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cen*
80	3.25	1	1.85	0.130	0.202	0.200	0.181	0.145	0.129		
82	2.1	3	1.85	0.129	0.207	0.193	0.193	0.174	0.134	0.120	
83	2.2	3	1.85	0.139	0.175	0.169	0.135	0.129	0.109	0.112	
84	3.1	1	1.85	0.087	0.103	0.104	0.101	0.104	0.104		
85	3.2	1	1.85	0.114	0.164	0.187	0.150	0.138	0.123		
86	3.1	2	1.85	0.128		0.246			0.150	0.133	1.68
87	3.6	2	1.85	0.128		0.185			0.133	0.138	5.65
88	2.9	1	1.85	0.123			0.151		0.118		
89	4.1	1	1.85	0.118			0.154		0.125		
90	2.4	2	1.85	0.132		0.192		0.164	0.134	0.122	2.30

TABLE II.

Effect of Administration of Hydrazine Sulfate (130 Mg. per Kilo) upon Glucose Tolerance of Rabbits.

Rabbit No.	Weight.	Fasting.	Blood sugar content.								Liver glycogen.	Remarks.
			Initial.	After hydrazine.	After injection of glucose (1.85 gm. per kilo).							
					1 hr.	2 hrs.	3 hrs.	4 hrs.	6 hrs.	8 hrs.		
	kg.	*days*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
78	2.7	1	0.134	0.153			0.367			0.241		Convulsions, stopped by glucose injection.
80	3.2	1	0.130	0.087	0.200		0.190		0.196		0.187	" "
84	3.0	2	0.087	0.093	0.153		0.154		0.121			
85	3.2	2	0.114	0.060	0.103	0.143		0.168	0.222	0.222	0.109	
88	2.9	2	0.123	0.105		0.174		0.176		0.150	0.183	
89	4.1	2	0.118	0.084		0.210		0.214		0.230	0.181	

zine, the hyperglycemia became more marked (Table II), with an average maximum increase of 90 per cent, and persisted for more than 6 hours as a rule. The glycogen deposited in the liver after glucose injection was very slight in the hydrazinized animal, the average content of the liver glycogen of four rabbits being 0.165 per cent. In both series of experiments the loss of glucose by excretion through the kidneys was found to be almost negligible, the maximum excretion being less than 10 mg. per hour. The difference in the hyperglycemia following the injection of glucose

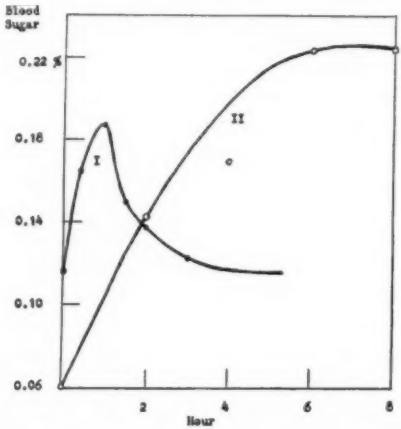

FIG. 1. A comparison of the glucose tolerance as shown by the variations in the glucose content of the blood following the subcutaneous injection of 1.85 gm. of glucose per kilo in the fasting animal (Rabbit 85) (Curve I) and in the same animal after hydrazine poisoning (Curve II).

in normal fasting rabbits and in hydrazinized rabbits is shown graphically in Fig. 1 in which the tolerance test for glucose is shown for Rabbit 85 before (Curve I) and after (Curve II) the administration of hydrazine.

Our observations concerning the hyperglycemia in normal and hydrazinized rabbits after the injection of glucose support the work of Underhill and Hogan (6) previously mentioned. The values for the glycogen of the liver are also in accord with those of Sato (10). 3 hours after the intravenous injection of 2.5 gm. of

glucose per kilo, the liver of rabbits which had undergone a preliminary period of inanition contained about 2 per cent of glycogen, while in control animals, similarly treated, but which received no glucose, the glycogen content was approximately 0.45 per cent.

Although it had previously been observed (6,9) that the glucose tolerance was diminished in hydrazine intoxication, no studies of the ability of the liver to form glycogen after glucose administration in hydrazine poisoning appear to have been made. We have demonstrated that, despite the persistent hyperglycemia after glucose administration in hydrazinized animals, the liver was unable to transform the excess glucose into glycogen. Hydrazine apparently so injures the hepatic cells that the glycogenetic function of the liver is deranged and this injury is manifested by the decrease in the glucose tolerance. This point of view is supported by the fact that a definite decrease in the tolerance for glucose is reported in hepatic injury, as in yellow atrophy produced by phosphorus poisoning (11). Clinical observations also lend support to this argument (12–17).

Since the power to synthesize glucose to glycogen was shown to be affected by hydrazine, it seemed of interest to determine whether the transformation of some simple carbon compound, normally a glucose former, to glucose was influenced under the conditions of hydrazine intoxication. The influence of hydrazine intoxication on the metabolic changes of lactic acid, which is closely related physiologically to glucose and also a probable intermediary product of the metabolism of alanine, was investigated.

That lactic acid may be converted to glucose in both depancreatized (18) and phlorhizinized (19) dogs has been long known and frequently confirmed (20–23). Röhmann (24) demonstrated that the administration of sodium or ammonium lactate to fasting rabbits led to glycogen storage in the liver. Perfusion experiments have also shown the possibility of glycogen formation from lactic acid in the turtle liver (20). Underhill and Blatherwick (25), who noted that the hypoglycemia following thyreoparathyroidectomy was relieved by the injection of calcium lactate, considered this effect to be due to the calcium ion rather than to lactic acid. Parnas and Wagner (26), who reported an interesting

case of hypoglycemia associated with a tumor of the liver in a young girl, observed that the oral administration of calcium lactate was effective in restoring the blood sugar temporarily to its normal level, and considered that the effect was due to the conversion of lactic acid to glucose. Pollak (27) did not observe any rise in blood sugar content after the subcutaneous injection of 1.3 gm. of lithium lactate into a rabbit of 2.2 kilos. This negative result is probably to be ascribed to the relatively small amount of lactate injected.

TABLE III.

Effect of Administration of Sodium Lactate on Sugar Content of Blood and on Glycogen Content of Liver.

Rabbit No.	Weight.	Fasting.	Sodium lactate as lactic acid per kilo.	Blood sugar content.					Liver glycogen.	Remarks.
				Initial.	After administration of sodium lactate.					
					1 hr.	3 hrs.	6 hrs.	9 hrs.		
	kg.	*days*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
90	2.6	2	5.0	0.143	0.211	0.231	0.174	0.154		Administration *per os.*
91	2.6	2	5.0	0.114	0.240	0.297	0.214*			" "
92	3.1	1	3.0	0.133	0.152	0.182		0.189*		" "
93*a*	2.8	2	3.0	0.144	0.157	0.191	0.200	0.108		" "
93*b*	2.7	3	3.0	0.114	0.150	0.186	0.178	0.123	1.85	Subcutaneous administration.
94	2.8	2	3.0	0.103	0.144	0.174	0.138	0.113		" "
95	2.8	4	3.0	0.116	0.135	0.158	0.167	0.129	2.09	" "
96	2.1	2	3.0	0.125	0.146	0.154	0.133	0.120		" "
97	2.2	2	3.0	0.101	0.120	0.118	0.121	0.110		" "

* Dyspnea, death.

The general plan of the present series of experiments was identical with that of the glucose series already presented. The lactic acid (inactive) was injected as the sodium salt, in 10 or 25 per cent solution, in doses usually equivalent to 3 gm. of lactic acid per kilo of body weight. As a rule the injection of this amount of sodium lactate resulted in the production of a hyperglycemia (Table III), the content of blood sugar reaching its maximum

(approximately 43 per cent higher than the normal figure) in about 3 hours and returning to the normal level within 9 hours. No abnormal symptoms were observed. A similar hyperglycemia followed the oral administration of the same amount of sodium lactate to two animals. One of these, however, showed marked depression and died 20 hours after the administration. Oral administration of larger doses (5 gm. per kilo) induced a more marked hyperglycemia, but was fatal in one of the two experiments, the rabbit dying in 6½ hours. In confirmation of the work of others, storage of glycogen was observed in the two animals killed for the glycogen determination (1.85 and 2.09 per cent of glycogen in the liver, Table III).

When sodium lactate was injected 6 hours after the previous injection of hydrazine, the magnitude and duration of the resultant

TABLE IV.

Effect of Hydrazine upon Hyperglycemia and Storage of Glycogen in Liver Following Subcutaneous Injection of Sodium Lactate.

Rabbit No.	Weight.	Fasting.	Hydrazine sulfate per kilo.	Sodium lactate as lactic acid per kilo.	Blood sugar content.						Liver glycogen.
					Initial.	After hydrazine.	After sodium lactate.				
							1 hr.	3 hrs.	6 hrs.	9 hrs.	
	kg.	*days*	*mg.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
94	2.8	3	130	3.0	0.103	0.154	0.151	0.200	0.185	0.177	0.092
96	2.1	3	130	3.0	0.125	0.115	0.145	0.181	0.185	0.139	0.022
97	2.2	3	130	3.0	0.101	0.084	0.110	0.176	0.143	0.143	0.760

hyperglycemia (Table IV) were greater than those observed in normal fasting rabbits, the average maximum increase of the blood sugar being 72 per cent and the duration of the hyperglycemia more than 9 hours. The glycogen content of the liver 10 hours after the injection of the lactate was low, especially in two of the three animals studied (Table IV).

The blood sugar curves following the injection of sodium lactate before and after the administration of hydrazine for Rabbit 96 are shown in Fig. 2 and need no further comment.

The storage of glycogen in the liver of normal fasting rabbits which we observed is comparable to that obtained by Röhmann (24). He fed rabbits which had previously fasted for 3 to 4 days

a few doses of 2.5 gm. of sodium lactate together with 2.5 gm. of sodium acetate (in order to prevent the oxidation of lactic acid) at intervals of a few hours and killed the animals 3 to 21 hours after the last administration. The livers of the animals which were fed the lactate contained about 1.86 per cent of glycogen, while control experiments in which no lactate was administered showed an almost complete lack of glycogen.

We have been unable to find any reference in the literature to the production of hyperglycemia in normal animals following the

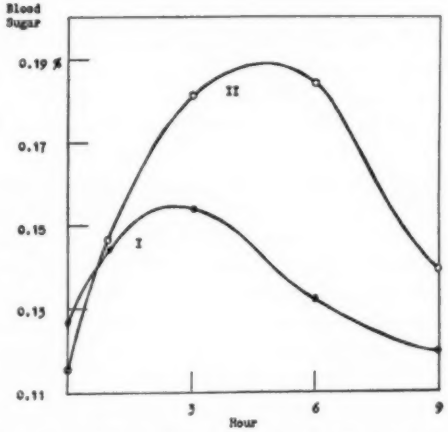

FIG. 2. The influence of hydrazine on the development of the hyperglycemia following the injection of sodium lactate (equivalent to 3 gm. of lactic acid per kilo) in Rabbit 96. Curve I shows the blood sugar values in the fasting period, and Curve II the values in hydrazine intoxication.

administration of lactate. Pollak's failure to observe any significant change after the subcutaneous injection of lithium lactate (27) may be due to the small amount injected, since the difference in the cations, *i.e.* lithium and sodium, can hardly explain the difference between his results and ours. Pollak injected lithium lactate in an amount equivalent to 0.55 gm. of lactic acid per kilo. We have observed that, as a rule, sodium lactate in doses less than 2.0 gm. as lactic acid per kilo did not induce any appreciable hyperglycemia.

The possibility was suggested that some oxidation product of lactic acid, *e.g.* pyruvic acid, with the power of reducing alkaline copper solutions might be formed in the organism and be estimated as glucose in the blood sugar determination. Blood after the administration of sodium lactate was tested for the presence of pyruvic acid by the method of Hurtley (28). It was not possible to detect pyruvic acid in any of the blood examined, although, in control tests, it was noted that positive tests could be obtained at a dilution of 1 part of pyruvic acid in 50,000 parts of the tungstic acid blood filtrate. These results make it improbable that the hyperglycemia observed was due to pyruvic acid.

These results obtained in the demonstration of the production of hyperglycemia and the formation of glycogen in the liver after the administration of sodium lactate to the normal animal furnish further evidence that lactic acid is a precursor of glucose in the organism.

Under like conditions a similar hyperglycemia was observed in hydrazinized animals. This indicates that hydrazine does not interfere, at least to any great extent, with the ability of the organism to transform lactic acid to glucose. The greater degree of hyperglycemia and its continuation over a longer period of time, observed in hydrazinized animals, indicate that, as previously shown, the ability to transform glucose to glycogen is greatly diminished by hydrazine. These conclusions are also supported by the experimental results of Underhill and Hogan (7). They observed that the glyoxalase activity of the liver of dogs was not altered appreciably by hydrazine. According to Dakin and Dudley (29), the conversion of lactic acid to glucose may be effected with the aid of the enzyme, glyoxalase, methyl glyoxal being formed as an intermediary product in this transformation.

$$C_6H_{12}O_6 \rightleftarrows CH_3.CO.CHO \rightleftarrows CH_3.CHOH.COOH$$

The activity of the glyoxalase in the liver and other tissues may be considered, therefore, as one of the indexes of the capacity of the animal organism to transform lactic acid to glucose.

It is characteristic of the hypoglycemias produced by insulin and by hepatectomy that convulsions result when an extremely low level of blood sugar is reached. These convulsions may be relieved and the animals restored to normal conditions for several

hours by the administration of glucose (30). In our studies of hydrazine poisoning, six of nine rabbits which received hydrazine sulfate in doses of 130 mg. per kilo developed convulsions. Immediately after the onset of the convulsions was observed, a 25 per cent solution of glucose (1.85 gm. per kilo) was injected subcutaneously. In four animals the administration of glucose produced no effect upon the convulsions and the animals died within a short time, while in the other two animals the convulsions ceased promptly after the injection of glucose. The animals were not restored, however, to the normal condition, but were usually depressed and finally became comatose and remained so, in one case, for more than 30 hours before death occurred. It is probable that, although the symptoms which are closely associated with hypoglycemia were relieved, death may have been due to the injury of functions of the liver other than those associated with carbohydrate storage, or to the injury of other organs produced by hydrazine. Williamson and Mann (31) observed that two dogs in a series of twenty-six, which received chloroform, developed convulsions which were relieved in one animal by glucose. Wierzuchowski (32) observed convulsions in three of ten dogs under the influence of phlorhizin and was able to relieve the convulsions within 20 minutes by the oral administration of glucose.

DISCUSSION.

On the Mechanism of the Hypoglycemia Produced by Hydrazine.

In general, hypoglycemia may be the result of two different types of reaction in the organism. (1) The decreased sugar content may be due to an increased rate of disappearance of sugar from the blood. This may be the result of (*a*) an increase in the combustion of glucose or glycolysis, (*b*) an increase in the rate of withdrawal of glucose for storage purposes or an increased rate of glycogenesis, or (*c*) an increased excretion of glucose or intermediary products of metabolism through the kidneys. (2) The lowered blood sugar may be the result of a decrease in the supply of sugar available in the system. Such a condition might arise either if glycogenolysis, the conversion of glycogen to glucose, is interfered with, or if glyconeogenesis, the conversion of non-carbohydrate materials to glucose, does not occur normally.

There is no evidence to show that the hydrazine poisoning induces an increased glycogenesis in the animal organism or that it facilitates the excretion of glucose or the intermediary products of carbohydrate metabolism through the kidneys. It was noted by Underhill (4) that after hydrazine injection the glycogen content of the liver and muscles markedly decreased and was never found to rise. Underhill and Kleiner (33) found no increased glucose or other reducing substances, and no lactic, hydroxybutyric, and diacetic acids or acetone bodies in the urine of hydrazinized dogs. We have not been able to demonstrate any increased excretion of glucose in hydrazinized animals following glucose administration, even when a marked hyperglycemia persisted as a result of glucose injections.

There is, however, evidence which has been interpreted as indicating an increased glycolysis after hydrazine administration (8). The respiratory quotient of fasting dogs increased slightly following the administration of hydrazine.[1] But this cannot be interpreted to be the result of an increased rate of glycolysis alone, rather than of changes in the character of the metabolic processes, because the following facts are not in accord with this assumption. (1) The basal metabolism of the dogs was not increased by the hydrazine (8). (2) The consumption of glucose by the surviving heart of the rabbit was not increased by the administration of hydrazine (5). (3) The utilization of the glucose which was parenterally introduced into the hydrazinized animal was never greater than that observed in the normal animal (6)

[1] Underhill and Murlin (8) state that "subcutaneously introduced dextrose is oxidized more rapidly in dogs that have received hydrazine than in the normal fasting animal." In support of this statement one fasting control experiment alone is cited (Dog 4). It should be noted that the conditions were not comparable, as the fasting animal, although heavier than the other animals, received the same amount of glucose. The fasting animal also fasted only 2 days while the hydrazinized dogs had fasted 13 days (10 days prior to hydrazine and 3 days after its injection) and 7 days (5 days prior to the injection of hydrazine and 2 days after). In a later experiment when this animal was injected with hydrazine and the rate of combustion of subcutaneously introduced glucose studied, the weight of glucose injected was double that previously injected for the fasting control. It is difficult to interpret these data because of this lack of adequate control experiments.

if the degree and duration of the hyperglycemia be made the criterion.

It would seem that the increased respiratory quotient, which was observed by Underhill and Murlin (8) in hydrazinized animals, was a manifestation of the hepatic injury caused by the poison. Porges (34) and Porges and Salomon (35) found that a rise in the respiratory quotient followed ligation of the abdominal aorta and the inferior vena cava below the diaphragm both in normal rabbits and in depancreatized dogs. Similar results were obtained by Rolly (36), Murlin, Edelmann, and Kramer (37), and others. Grafe and Denecke (38) and Mann (39) also observed that the respiratory quotient was usually raised after the total extirpation of the liver in dogs. Porges considered that the fact was the result of increased carbohydrate combustion, fat and protein being more difficultly oxidized in the absence of the liver; while Rolly and Murlin and his coworkers, who demonstrated that there was always a fall in the carbon dioxide content of the blood whenever the respiratory quotient of the operated animal was increased, considered that the increase was the result of pulmonary overventilation caused mechanically by the effect of the operation and not the result of an alteration in the character of the metabolism as thought by Porges. In hydrazine poisoning, as shown by Hendrix and McAmis (40), the pH and carbon dioxide combining-power of the blood is always increased, and, therefore, the increase in the respiratory quotient of the animal after the administration cannot be considered solely as the result of the pulmonary overventilation.

An explanation of the mechanism of the hydrazine hypoglycemia must, therefore, be sought in the second type of reaction; *i.e.*, the decrease in the supply of glucose available for combustion. There is no evidence that glycogenolysis is diminished, since the glycogen content of the liver after glucose injection is less in hydrazinized animals than in normal fasting animals. There are, however, reasons to believe that the protein metabolism is deranged and that the normal formation of sugar from protein (amino acids), the most important type of glyconeogenesis, is diminished quantitatively in hydrazine intoxication (1). Moreover, the occurrence of lipemia in hydrazine poisoning (41) indicates a derangement in the normal metabolism of fat.

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The series of reactions which are suggested to occur in hydrazine poisoning is somewhat as follows. Hydrazine injures the hepatic cells and induces conditions under which the transformation of non-carbohydrate materials into glucose and glycogen (glyconeogenesis), as well as the synthesis of glycogen from glucose and related substances, does not occur normally. Under these conditions the glycogen store of the liver, which is constantly depleted by glycogenolysis, cannot be maintained from the fat and protein stores of the organism and its amount is greatly diminished. A large amount of fat is mobilized from the storage depots and enters the liver cells, producing the lipemia and fatty infiltration of the liver commonly observed in hydrazine poisoning. As a result of hepatic injury, the normal deamination of amino acids does not occur and hyper amino acidemia results with the consequent failure of glyconeogenesis. Under these conditions, the fat and protein metabolism being disturbed, the organism utilizes chiefly carbohydrate, and this change in the character of the metabolism manifests itself in the increased respiratory quotient. The increase in the consumption of carbohydrate together with the limited supply of carbohydrate available as a result of diminished glyconeogenesis eventually produces the hypoglycemia characteristic of hydrazine poisoning.

SUMMARY.

1. Glycogen synthesis did not take place in the liver of hydrazinized rabbits, despite the marked hyperglycemia which followed the injection of glucose. This derangement of the glycogenetic function of the liver is considered to be the cause of the decreased glucose tolerance of rabbits poisoned with hydrazine.

2. The administration of sodium lactate to rabbits was found to induce a development of hyperglycemia and an accumulation of glycogen in the liver. The hyperglycemia produced by sodium lactate was intensified in its magnitude and duration by the previous injection of hydrazine, while the synthesis of the liver glycogen from sodium lactate was markedly diminished under these conditions.

3. Convulsions and other symptoms similar to those following hepatectomy were often observed in the rabbit during hydrazine

poisoning. In some cases the convulsions were immediately alleviated by the injection of glucose.

4. The mechanism of the hypoglycemia produced by hydrazine is discussed. It is suggested that the primary cause is failure of normal glyconeogenesis, the transformation of non-carbohydrate materials to glucose, as a result of which the supply of glucose available is diminished because of the hepatic injury produced by hydrazine.

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SOME OBSERVATIONS BEARING ON THE RÔLE OF ACETALDEHYDE IN ANIMAL METABOLISM.

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In his recent review on the intermediary metabolism of the carbohydrates, Shaffer (1) has discussed certain evidence which has been interpreted to indicate that glucose metabolism in the animal body follows much the same course as in yeast fermentations. Some of the evidence given special prominence includes the work of Neuberg (2) who showed the importance of pyruvic acid and acetaldehyde in the fermentation of glucose by yeasts, the finding by Stepp and Feulgen (3, 4) of traces of acetaldehyde constantly present in normal and pathological urines, and the studies of Friedmann (5) and of Embden and Oppenheimer (6) which indicate that acetaldehyde and pyruvic acid have a common fate in the surviving liver.

In view of these findings and on account of the volatile nature of acetaldehyde, it seemed of interest to study the excretion in expired air, as well as in urine, of acetaldehyde under normal and certain experimental conditions, especially after administration of acetaldehyde, ethyl alcohol, and pyruvic acid. Since the question of acetoacetic acid formation from acetaldehyde is involved, an examination for this substance was also made.

I. Excretion of Acetaldehyde Injected into Peritoneal Cavity.

Measured amounts of acetaldehyde solutions in normal saline were slowly injected into the peritoneal cavity of rabbits. No

* The work on acetaldehyde excreted in normal and diabetic urines was done for the most part in the Department of Biological Chemistry of Washington University at the suggestion of Prof. Philip A. Shaffer.

anesthetic was necessary. Expired air was collected during the injection and for 2 hours following by means of a device made from a large tin can, one end of which contained inlet[1] and outlet tubes for a current of air, and the other end replaced by a perforated rubber membrane which was slipped over the head of the rabbit. Expired air was carried away by suction and passed through two large aeration tubes containing potassium bisulfite solution. Sulfite bound by acetaldehyde was determined iodometrically as in the Clausen lactic acid method (7). Urine was collected if passed during an experiment in a pan underneath the animal board. At the end of an experiment the animal was placed in a cage on a wire grating over a collecting basin into which the bladder was promptly emptied in several of the experiments. If the bladder was not voluntarily emptied within a few minutes, urination was induced by external pressure over the bladder. For the determination of acetaldehyde the acidified urines were aerated in tall cylinders, and the acetaldehyde collected in bisulfite and titrated iodometrically as above.

With the question of acetoacetic acid formation in mind the urines after aeration were transferred to a distilling flask, diluted to a volume of about 100 cc., and from this 25 cc. were distilled into 20 cc. of 0.02 N $KHSO_3$. The sulfite bound was titrated in the usual manner. This quantity was always small and probably due to a residue of acetaldehyde in the aerated urines, since whatever it was due to was subsequently destroyed by peroxide in the procedure used by Clausen (7) to differentiate between acetone and acetaldehyde in the products of the first titration. No evidence was observed therefore of acetoacetic acid formation.

The question of acetaldehyde elimination has been studied by at least two previous workers. Albertoni (8) found a large percentage of acetaldehyde administered to dogs eliminated in expired air and urine, and remarked on the ease with which so unstable a substance as acetaldehyde is able to pass through the organism unchanged. Reizenstein (9) in similar experiments found a smaller percentage eliminated in urine and only doubtful traces in expired air.

The first five experiments in the accompanying table indicate

[1] The inlet tube communicated with outside air.

that acetaldehyde is hardly an inert substance but that small amounts do always escape and that more is eliminated in expired air than in urine.

II. Excretion of Acetaldehyde Following Administration of Pyruvic Acid.

Quantities of pyruvic acid[2] varying from 3 to 7 gm., dissolved in 150 cc. of water and made alkaline to methyl red with sodium

TABLE I.

Excretion of Acetaldehyde Following Administration of Acetaldehyde, Pyruvic Acid, and Ethyl Alcohol.

Experiment No.	Subject.	Substance administered.	Route of administration.	Acetaldehyde in expired air.	Acetaldehyde in urine.
				mg.	*mg.*
1	Rabbit.	Acetaldehyde, 600 mg.	Intraperitoneal.	1.4	0.41
2	"	" 100 "	"	0.2	Nil.
3	"	" 300 "	"	0.3	0.10
4	"	" 1600 "	"	4.5	1.52
5	"	" 2500 "	"	8.7	3.3
6	"	Pyruvic acid, 3 gm.	"	Nil.	Nil.
7	"	" " 6 "	"	"	"
8	"	" " 4 "	"	"	"
9	"	" " 7 "	"	"	"
10	"	Ethyl alcohol, 6 gm.	"	0.14	0.04
11	"	" " 8 "	"	0.25	0.13
12	"	" " 10 "	"	0.31	0.19
13	A.P.B.	" " 25 "	Oral.	0.10	0.03
14	"	" " 50 "	"	0.36	0.19

hydroxide, were injected slowly over an interval of about an hour into the peritoneal cavity of rabbits.[3] Expired air and urine were collected during the injection and for 2 hours following, and examined for acetaldehyde as described in the preceding section.

[2] Freshly prepared by Mr. Milton Levy of the Department of Biological Chemistry.

[3] These animals at the close of the experiment were very much intoxicated in contrast to those after the acetaldehyde injections. In three of the experiments reported the animals were dead the following morning. Autopsy examinations were not performed.

As indicated in Table I no acetaldehyde was found to be eliminated during this interval. That pyruvic acid was well absorbed is certain from the fact that a considerable amount of unchanged pyruvic acid was found in the urine in each experiment after aeration.[4]

Since pyruvic acid has been assumed to be the precursor of acetaldehyde in animal metabolism as well as in yeast fermentation, it might be expected that traces of acetaldehyde should have escaped in these experiments, as in the experiments where small amounts of acetaldehyde were administered. Although none was found, it is conceivable that acetaldehyde as it is formed in metabolism, nascent so to speak, is very rapidly converted to acetic acid or some other metabolite. This point is of special interest in connection with the experiments on alcohol administration in the following section.

III. Excretion of Acetaldehyde Following Administration of Ethyl Alcohol.

In Experiments 10, 11, and 12 ethyl alcohol was slowly injected with 150 cc. of water into the peritoneal cavity of rabbits. Expired air and urine were collected and examined for acetaldehyde as in the two previous sections. In Experiments 13 and 14, on subject A. P. B., the alcohol was taken by mouth with 300 cc. of water. Collection of expired air in these last two experiments was accomplished by fitting a mouthpiece in the perforated rubber membrane of the respiration apparatus. A rubber balloon was also connected in the circuit leading to the absorption tubes in order that expirations of larger volumes might be temporarily accommodated.

Small amounts of acetaldehyde as shown in Table I were always found in both expired air and urine in each of the alcohol experiments. That these quantities were well outside the limits of titration is evident when one considers that 0.1 mg. of acetaldehyde is equivalent to about 5.0 cc. of 0.001 N iodine.

The excretion of definite quantities of acetaldehyde where it does occur as a metabolite lends additional significance to the

[4] Pyruvic acid was tested for with nitroprusside as for acetoacetic acid; a blue color which on standing changed to violet was obtained. The urines obtained after injection of acetaldehyde failed to give this test.

ne gative results obtained after the administration of pyruvic acid. It would also make it seem more likely that acetaldehyde should be constantly excreted in detectable amounts by the lungs as well as the kidneys if it were one of the chief intermediates in carbohydrate metabolism.

The urines of the alcohol experiments on rabbits were also examined for acetoacetic acid as follows:[5] After aeration they were transferred to a distilling flask and diluted to a volume of about 100 cc. From this 25 cc. were distilled into a flask containing 20 cc. of 0.05 N $AgNO_3$. To this were added 9 cc. of 0.1 N NaOH and 15 cc. of 0.1 N NH_4OH. The flask was then stoppered and set away in the dark for 24 hours. At the end of this time it was acidified with sulfuric acid, diluted to 100 cc., and from this 25 cc. were distilled into 20 cc. of 0.02 N $KHSO_3$ solution. The subsequent titrations for acetone-bound sulfite were in each case zero.

The urines of the alcohol experiments on man were handled slightly differently as follows: The urines when passed were collected in a distilling flask, acidified, and one-tenth of the volume immediately distilled into 20 cc. of 0.02 N $KHSO_3$. The sulfite bound was titrated as usual. The products of titration were then transferred to a distilling flask, an excess of $CaCO_3$ added, and 25 cc. of distillate collected in a flask containing 20 cc. of 0.05 N $AgNO_3$. This was made ammoniacal and examined for acetone as in the rabbit urines above. These urines also gave negative results for acetone. As in the urines of the acetaldehyde experiments, acetaldehyde was excreted but no evidence of acetaldehyde condensation as observed by Friedmann (5) in the perfused liver was found.

IV. Normal Excretion of Acetaldehyde.

By means of a technique substantially the equivalent of that described by Fricke (10) a volatile substance which reduces am-

[5] Ammoniacal silver was used to destroy acetaldehyde not removed by aeration rather than peroxide because blank experiments showed that when a mixture of alcohol and acetaldehyde, such as might be expected in these distillates, was treated with sodium peroxide that aldehyde was formed from the alcohol as fast as it was destroyed; *i.e.*, the subsequent distillates contained as much or more sulfite-binding substance as the original mixture. Under the conditions described above the destruction of acetaldehyde was found to be nearly complete and acetone only slightly attacked.

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moniacal silver has been found in normal as well as in diabetic urines,[6] confirming to this extent the results of Stepp and Feulgen (3, 4). From 4 liters of urine silver reductions equivalent to from 1 to 3 mg. of acetaldehyde have been observed. That this is chiefly not due to acetaldehyde however seems likely since it has not been possible to get a positive test for acetaldehyde with dimethylhydroresorcinol in the distillates of from 4 to 8 liters of urine from the same groups of individuals. The technique of Stepp and Feulgen (3) which was employed gives a voluminous precipitate with quantities of acetaldehyde as small as 1 mg. in a volume of 25 cc. Moreover, if such amounts of acetaldehyde were being excreted constantly in urine it should be possible to detect it in expired air, since in the first part of this paper it was observed that following the administration of acetaldehyde as well as after ethyl alcohol, greater quantities of acetaldehyde were found in expired air than in urine. This point has been tested over two 12 hour periods and one 24 hour period as follows: Expired air from the subject A. P. B. was led through sulfite solution as in the alcohol experiments. At the end of a 2 hour interval the sulfite solution was changed and another 2 hour collection made when convenient. In two tests, six of these 2 hour collections were united in a distilling flask, treated with excess $CaCO_3$, diluted to 300 cc., and from this 30 cc. were distilled into 20 cc. of 0.02 N $KHSO_3$. The distillate was examined for acetaldehyde by the usual iodometric titration. In the other test twelve of the 2 hour periods were united and similarly tested for acetaldehyde. In each case the titrations[7] indicated doubtful traces of acetaldehyde.

SUMMARY AND CONCLUSIONS.

The excretion of acetaldehyde in expired air and urine has been studied under normal conditions and after the administration of acetaldehyde, ethyl alcohol, and pyruvic acid.

[6] The quantities found in diabetic urines were of the same order as those found in normals.

[7] A small amount of a sulfite-binding substance was found, but since it was only slightly destroyed by the usual peroxide treatment it was probably acetone.

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Although a questionable reducing substance was found present in normal as well as in diabetic urines, none was found in expired air.

Following the intraperitoneal administrations of acetaldehyde to rabbits, it appears in appreciable amounts in both expired air and urine.

Following the administration of ethyl alcohol acetaldehyde was likewise found in both expired air and urine, but none was found following the administration of pyruvic acid.

These results furnish no evidence in favor of the view that acetaldehyde is an important intermediate in carbohydrate metabolism of higher animals.

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THE SPECIFIC DYNAMIC ACTION OF GELATIN HYDROLYSATES.

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INTRODUCTION.

Lusk (1) has established the fact that the amino acids glycine and alanine have a powerful stimulating action on the metabolism, and evolved the theory that the specific dynamic action of protein was due chiefly to these amino acids. Working in Professor Lusk's laboratory, the writer (2) investigated the specific dynamic action of certain proteins of varying origin and of widely differing content of glycine and alanine. It was found, contrary to *a priori* considerations, that the heat-stimulating properties of these proteins were the same. The apparent contradiction of Lusk's theory led to further work (3), with a view to the elucidation of this anomaly. The possibility existed that the specific dynamic action, for example, of casein, which contains no glycine, might be due to the synthesis of the latter substance when casein was ingested. Increasing the quantity of ingested glycine increases the heat production proportionately, as was shown by Lusk (4). If, when casein was ingested, glycine were synthesized, the ingestion of glycine together with casein might be expected to result in a summation of specific dynamic action. It was found, however, that no summation occurred. This was not due to suppression of the glycine synthesis, for Csonka (5) has shown that in the pig there is less glycine eliminated after the administration of casein than following the ingestion of gelatin. Moreover, this lack of summation was also evident when gelatin and glycine, or gelatin and alanine, were given together, in spite of the fact that gelatin, in the quantity administered, contained 26 per cent

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glycine and 4 per cent alanine. From these and other considerations, it was concluded that there existed a difference between the metabolism of proteins and of individual amino acids. The present research was instituted in order to see whether a similar difference existed between the individual amino acid—in this case glycine—and the *in vitro* hydrolysates of a protein. The results obtained indicate that the *in vitro* hydrolysates behave, in respect to their stimulating effect upon oxidations, like their parent protein, and not like their constituent amino acids.

Method.

The apparatus used was essentially that described by Benedict and Homans (6). The system was a closed one, and the air was circulated by means of a rotary blower. CO_2 was absorbed by soda lime and water by sulfuric acid. Oxygen was admitted from a tank, which was weighed before and after the experimental period. The animal chamber was kept at a temperature of about 25° by a water bath, and the variations in the temperature of the outgoing air rarely varied by more than 0.1°. All the movements of the animal within the chamber were recorded by changes of the pressure in a series of rubber tubes covered by a cloth, upon which the animal lay, these pressure changes being transmitted to a tambour and so to a kymograph. Any experimental period during which an appreciable amount of movement occurred was discarded. The experiments were usually carried out through the 5th hour after the administration of the food to be tested.

The capacity of the animal chamber was about 40 liters, and the ventilation was approximately 35 liters per minute. This ventilation was sufficient so that the changes in the residual air of the box were quite small. In the case of water vapor, they did not exceed a maximum of 0.1 gm. per hour, which would, if neglected, have entailed an error of about 1.5 per cent; while the changes in the CO_2 content of the box were even less—never more than 0.05 gm. and usually 0.01 to 0.02 gm. Nevertheless, it was thought advisable to sample the residual air and to make the necessary corrections. As in all such systems, the errors fell almost entirely on the oxygen, for this was determined by weigh-

ing the amount of oxygen required in an experimental period to keep the volume constant, after correcting for the CO_2 and water in the residual air and for changes in temperature and barometric pressure. The apparatus, as shown by alcohol checks, to which reference will be made later, was accurate to 1.9 per cent.

The experiments were carried out on a single animal, maintained on a constant diet consisting of 75 gm. of cracker meal, 75 gm. of beef heart, 15 gm. of lard, and 5 gm. of bone ash. On this diet the animal maintained a fairly constant weight of about 6.5 kilos. In order to make the gelatin administered in certain experiments more palatable, 2 gm. of Liebig's extract were added. Lusk has shown that its specific dynamic action is neglible. However, to be consistent, it was added to the hydrolysates as well.

Preparation of the Hydrolysates.

The protein used was gelatin, and both an acid and a trypsin hydrolysate were made. The acid hydrolysate was prepared by boiling 250 gm. of gelatin with 300 gm. of H_2SO_4 and 650 cc. of water until the biuret reaction could no longer be obtained—usually 48 to 60 hours. At first neutralization was attempted with $Ba(OH)_2$, but it was found difficult to remove the barium salts of the amino acids. This resulted in a sufficiently toxic action so that the animal was prone to vomit, or at least was restless and uncomfortable. It was found that neutralization by $Ca(OH)_2$ obviated this difficulty. The reaction of the final product was faintly acid to litmus. As a rule the animal could be persuaded to lap up the mixture, but occasionally it was necessary to resort to the stomach tube.

The trypsin hydrolysate was prepared by treating 150 gm. of gelatin with 15 gm. of trypsin (Difco) in 3 liters of 0.5 per cent Na_2CO_3 and letting the mixture stand at 37° under toluene for 10 days, at the end of which time 6 gm. more of trypsin were added, and the hydrolysis allowed to go on for 5 days longer. Frankel (7) has found that under these conditions between 58 to 67 per cent of a protein is carried beyond the peptide form. The trypsin hydrolysate in the present experiments always gave the biuret reaction.

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Methods of Calculation.

The methods of calculation were the same as those discussed in the papers by Rapport (2) and by Weiss and Rapport (3). Glycine was calculated on the basis of the assumed actual metabolism of this amino acid when given alone, derived from Csonka's (8) figures on the rapidity of the sugar excretion following the ingestion of glycine in phlorhizin diabetes. When glycine was given together with a protein hydrolysate, the usual calculations based on the N excretion during the period of the experiment were made, involving, as has been shown (3), an inappreciable error.

TABLE I.
Basal Metabolism.

Date.	Experiment No.	R.Q.	Calories per hr.
Mar. 22	1	0.83	13.97
" 27	6	0.81	13.70
Apr. 9	11	0.81	13.38
" 16	13	0.86	13.62
Average		0.83	13.67

EXPERIMENTAL.

A. Basal Metabolism.

Lusk and DuBois (9) have shown the constancy of the basal metabolism in a series of eleven dogs. The basal heat production of the dog used in the present experiments was determined on four occasions during the duration of the experiments (Table I). The average metabolism was 13.67 calories with a maximum variation from this mean of +0.3 calories and −0.29 calories or ±2.2 per cent. The constancy of the basal heat production may, therefore, fairly be said to be almost within the limits of error of the apparatus. The maximum deviation (from 13.38 to 13.97) involved an error of 4.3 per cent.

The phenomenon of an initial decrease in metabolism due to cage life, which was pointed out by Lusk (4), was observed also in this animal, the heat production dropping about 20 per cent

during the first 5 weeks of the dog's stay in the laboratory, in spite of an actual gain in weight amounting to 0.4 kilos.

TABLE II.

Ingestion of Gelatin with Its Hydrolysates.

Average per hour of 4 hour period.

Food.	Experiment No.	Date.	Urinary N.	CO_2	O_2	R.Q.	Calories.	Increase over basal.
			gm.	*gm.*	*gm.*			*per cent*
Gelatin (5 gm. N).....	2	Mar. 23	0.493	6.84	6.14	0.81	19.63	43.6
" (5 " ").....	5	" 26	0.498	6.63	5.75	0.84	18.48	35.2
Average.....								39.4
H_2SO_4 hydrolysate of gelatin (5 gm. N).	3	Mar. 23	0.457	6.99	5.88	0.86	19.20	40.5
	9	" 31	0.466	7.19	5.93	0.88	19.34	41.6
	18	May 8	0.457*	6.90	5.75	0.87	18.76	37.2
Average.....								39.8
Gelatin (2.5 gm. N) and H_2SO_4 hydrolysate (2.5 gm. N).	4	Mar. 25	0.441	6.60	5.74	0.84	18.63	36.3
	19	May 10	0.461	6.88	5.88	0.85	19.15	40.1
Average.....								38.2
Trypsin hydrolysate of gelatin (5 gm. N).	10	Apr. 8	0.596	7.21	6.21	0.84	19.87	45.4
	20	May 11	0.542	7.46	6.28	0.86	20.09	47.0
Average.....								46.2
Gelatin (2.5 gm. N) and trypsin hydrolysate (2.5 gm. N).	14	Apr. 21	0.526	6.99	6.20	0.82	19.94	45.9
	15	" 22	0.480	7.18	6.27	0.84	20.31	48.6
Average.....								47.3

* Urine not obtained. N assumed to be the same as in Experiment 3.

B. Ingestion of Gelatin with Its Acid Hydrolysate.

Following the feeding of a quantity of gelatin yielding 5 gm. of nitrogen, the heat production rose in two experiments to 19.63

calories (or 43.6 per cent increase over the basal) and 18.48 calories (or 35.2 per cent increase) respectively. The average increase in metabolism was 39.4 per cent.

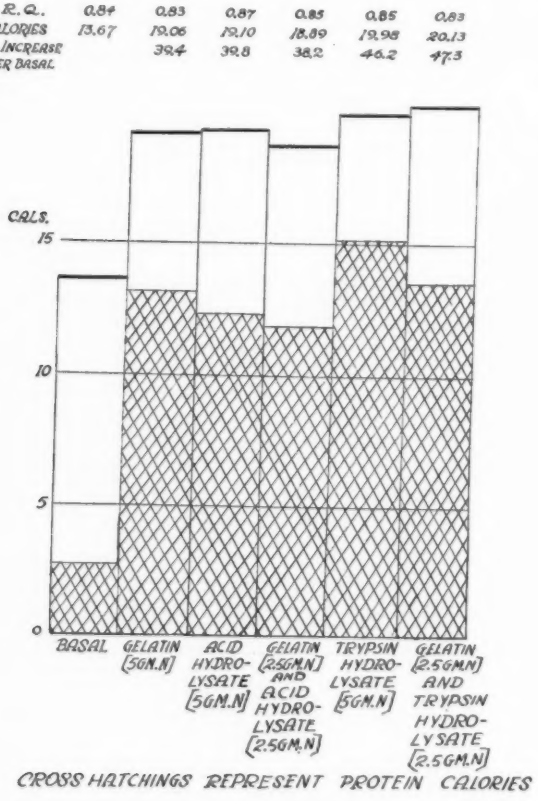

CHART I. Ingestion of gelatin with its hydrolysates.

The rise in metabolism following the ingestion of the acid hydrolysate of gelatin, also containing 5 gm. of N, was almost identical, amounting to 40.5, 41.6, and 37.2 per cent, respectively, in three experiments, the average increase being 39.8 per cent (Table II, Chart I).

When half portions of each of these foodstuffs were administered together, there resulted an increase in metabolism, in two experiments, of 36.3 and 40.1 per cent, showing an average elevation of 38.2 per cent.

There was thus a complete summation of the effects of the two substances.

C. Ingestion of Gelatin with Its Trypsin Hydrolysate.

After an amount of the trypsin hydrolysate containing 5 gm. of N was ingested, the metabolism rose, on two occasions, to 45.4 and 47.0 per cent above the basal level, the average increase being 46.2 per cent. When half portions of gelatin and trypsin hydrolysate were given together, there was again a complete summation, for the metabolism rose, in one case to 45.9 per cent, in another to 48.6 per cent above the basal, an average of 47.3 per cent. The trypsin hydrolysate appeared to offer a slightly greater stimulus to metabolism than either the gelatin or the acid hydrolysate. The difference was small, but consistent.

It is clear, therefore, that *when a protein is administered with the products of its in vitro digestion, the specific dynamic action of the two is summated. This is true whether the hydrolysis is carried to completion, as in the case of the acid hydrolysate, or is only partially complete, as in the instance of hydrolysis by trypsin.* In this respect, the hydrolysates behave like the protein itself, for, as was shown by Williams, Riche, and Lusk (10) and confirmed by the writer (2), the specific dynamic action of a protein increases as the amount metabolized increases. Had the hydrolysates acted in a manner similar to that of their contained glycine and alanine, their specific dynamic action, as was shown by Weiss and Rapport (3), would not have been summated.

D. Ingestion of the Hydrolysates with Glycine.

The specific dynamic action of 10 gm. of glycine was tested in one experiment, and the increase over the basal amounted to 27.7 per cent through the 5th hour after administration. This is comparable with the rise obtained after the same amount of glycine in a larger dog (4, 3).

The acid hydrolysate had increased the metabolism 39.8 per

cent. When the same quantity was given with 10 gm. of glycine, the resulting rise in the heat production was 38.2 (Table III, Chart II). There was a complete absence of summation.

For some reason, the dog retained a mixture of glycine and the trypsin hydrolysate with difficulty. The experiment was at-

TABLE III.

Administration of Glycine with the Hydrolysates of Gelatin.

Average per hour of 4 hour period.

Food.	Experiment No.	Date.	Urinary N.	CO_2	O_2	R.Q.	Calories.	Increase over basal.
			gm.	*gm.*	*gm.*			*per cent*
Glycine (10 gm.)	7	Mar. 29	0.382	6.29	5.24	0.87	17.46	27.7
H_2SO_4 hydrolysate. (Average of 3 experiments.)			0.462	7.03	5.85	0.87	19.10	39.8
Glycine (10 gm.) (by mouth) and H_2SO_4 hydrolysate (5 gm. N).	8	Mar. 30	0.596	7.37	5.91	0.91	18.89	38.2
Glycine (10 gm.) (intravenously) and H_2SO_4 hydrolysate (5 gm. N).	17	May 7	0.436	6.88	5.62	0.89	18.38	34.5
Trypsin hydrolysate. (Average of 2 experiments.)			0.569	7.34	6.25	0.85	19.98	46.2
Glycine (10 gm.) and trypsin hydrolysate (5 gm. N)	12	Apr. 14	0.612	8.07	6.66	0.88	21.42*	56.7*
Glycine (10 gm.), gelatin (2.5 gm. N), and trypsin hydrolysate (2.5 gm. N)	16	Apr. 28	0.524	7.80	6.35	0.89	20.65	51.1

* 3 hour experiment. See text.

tempted four times. On three occasions, it vomited during the 1st or 2nd hour of the experimental period. On the fourth occasion, it retained the food over 4 hours (through the 3rd hour of the experimental period) but finally vomited during the last hour. The first 3 hours of this experiment were calculated, as the recording apparatus showed no unusual evidence of movement.

The average of these hours showed an increased metabolism of 56.7 per cent, as compared with 46.2 per cent when the trypsin hydrolysate alone was administered. The metabolism was drop-

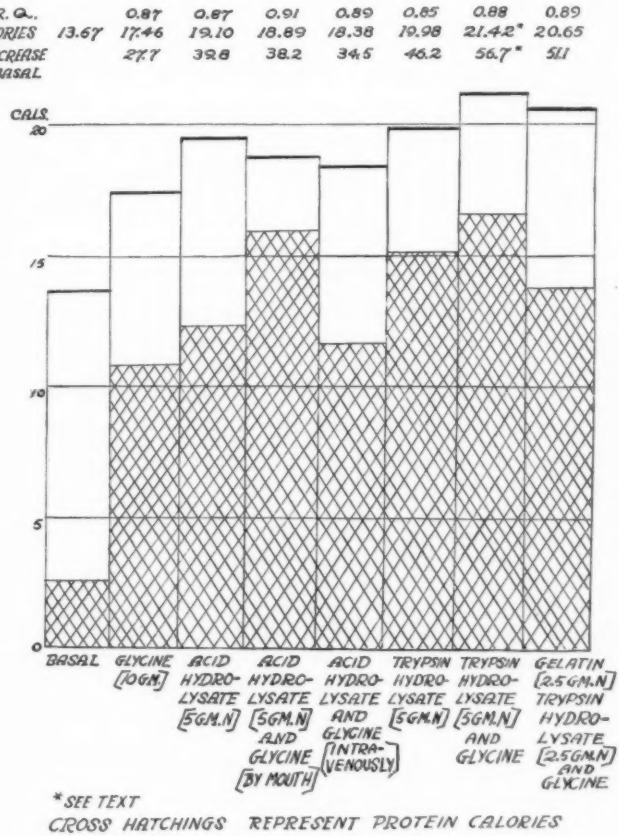
R.Q.
CALORIES 13.67 17.46 19.10 18.89 18.38 19.98 21.42* 20.65
% INCREASE OVER BASAL 27.7 39.8 38.2 34.5 46.2 56.7* 51.1
0.87 0.87 0.91 0.89 0.85 0.88 0.89
CALS.
20
15
10
5
0
BASAL
GLYCINE (10 GM.N)
ACID HYDROLYSATE (5 GM.N)
ACID HYDROLYSATE (5 GM.N) AND GLYCINE (BY MOUTH)
ACID HYDROLYSATE AND GLYCINE (INTRAVENOUSLY)
TRYPSIN HYDROLYSATE (5 GM.N)
TRYPSIN HYDROLYSATE (5 GM.N) AND GLYCINE
GELATIN (2.5 GM.N) TRYPSIN HYDROLYSATE (2.5 GM.N) AND GLYCINE
*SEE TEXT
CROSS HATCHINGS REPRESENT PROTEIN CALORIES

CHART II. Administration of glycine with the hydrolysates of gelatin.

ping rapidly by the 3rd hour, and experience leads one to the belief that during the last hour, if the animal had remained quiet, the heat production under these conditions would have been, if

anything, less than that of the 3rd hour. Assuming it to have been the same, the average of the 4 hours would have shown an increase in metabolism of 53.4 per cent, or very little more than that following the ingestion of the hydrolysate alone.

The inexplicable evidence of irritation as a result of feeding the mixture makes it seem plausible that in spite of the lack of movement, the animal was alert and uncomfortable, and it is probable that the slightly increased metabolism was due to this cause (see (2) p. 507). In any case, there is certainly no indication of complete summation, and it is concluded that the trypsin hydrolysate showed an absence of summation with glycine similar to that seen when the amino acid was given with the acid hydrolysate.

E. Ingestion of Gelatin, Trypsin Hydrolysate, and Glycine.

When half portions of gelatin and trypsin hydrolysate, which together had caused a rise in metabolism of 47.3 per cent, were given together with 10 gm. of glycine, the resulting increase was only 51.1 per cent (Table III). The difference between these two results was within the limits of error. *The mixture of gelatin and its hydrolysate, like either of these two substances alone, neutralized the stimulating action of glycine.*

F. Administration of Acid Hydrolysate by Mouth and Glycine Intravenously.

In the experiments reported by Weiss and Rapport (3), the question arose as to whether the observed effects might not be due to a change in the rate of absorption from the alimentary canal. It was found in those experiments that the method of administering glycine, whether by mouth, subcutaneously, or intravenously, did not affect its specific dynamic action, and that, moreover, its lack of summation with a protein was as evident after parenteral injection as after ingestion by mouth. The same was found to be the case as regards the absence of summation with the acid hydrolysate. When the latter and glycine were given together by mouth, the increase in heat production was 38.2 per cent; when the glycine was injected intravenously immediately following the ingestion of the acid hydrolysate, the resulting rise in metabolism was 34.5 per cent (Table III). In

both cases there was no evidence whatever of summation of effect. *We are justified, therefore, in saying that, whatever may be the factors that annul the specific dynamic action of an amino acid when it is given with a protein hydrolysate, they are capable of acting outside of the gastrointestinal tract.*

Nitrogen Elimination.—As in the experiments of Weiss and Rapport, the nitrogen elimination bore no constant or significant relation to the height of the metabolism, and hence throws no light upon the phenomena reported. The possible reasons for this have been sufficiently discussed in the paper referred to. (The N elimination data are given in Tables II and III.)

TABLE IV.

Alcohol Checks.

Date.	Experiment No.	Calories.	R.Q.
Mar. 17	3	12.00	0.667
Apr. 5	4	15.46	0.673
" 5	5	17.51	0.670
May 12	6	13.32	0.680
Average		14.57	0.673

Mean error = 0.9 per cent.
Maximum error = 1.9 " "

G. Alcohol Checks.

The validity of the data presented in this paper is attested by the results of the alcohol checks presented in Table IV.

SUMMARY.

1. When gelatin together with either its acid or its trypsin hydrolysate is administered to a dog, the specific dynamic action of the two substances is summated.

2. When either of these hydrolysates is given to the animal together with glycine, the specific dynamic action of the latter is neutralized, just as when the protein itself is administered with glycine.

3. That this neutralization of the stimulating action of glycine can occur outside of the gastrointestinal tract is shown by the

fact that it occurs when the glycine is injected intravenously immediately after the taking of the hydrolysate by mouth.

4. When gelatin, the trypsin hydrolysate, and glycine are ingested together, there is summation of the specific dynamic action of the protein and the hydrolysate and at the same time an inhibition of the stimulatory action of the glycine.

5. The evidence indicates that when a protein is hydrolyzed *in vitro*, the properties that cause its peculiar specific dynamic action remain those of the protein itself and are to be differentiated from the heat-stimulating properties of its constituent amino acids.

6. As in the case of the intestinal digestion products of a protein, the *in vitro* hydrolysates yield substances that are capable of neutralizing the specific dynamic effect of glycine, but do not neutralize the glycine contained in the protein itself.

It remains to investigate the nature of these substances, a preliminary to which would be the isolation, if possible, of the particular fraction or fractions of the protein digestion products in which they lie. Such an investigation is being undertaken.

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XUM

A STUDY OF THE DISTRIBUTION OF THE DIFFUSIBLE AND NON-DIFFUSIBLE CALCIUM IN THE BLOOD SERA OF NORMAL ANIMALS.

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Within the last few years the literature on the state of calcium in blood serum has been reviewed by a number of authors (1–3). It seems inexpedient, therefore, to undertake an exhaustive survey of the literature at this time, but we will summarize in a few paragraphs the present status of the problem in so far as it is pertinent to this investigation. Since the pioneer experiments of Rona and Takahashi (4) in 1911 the results of both compensation dialysis and ultrafiltration experiments on the sera of a considerable number of normal mammals including man have shown from 55 to 75 per cent of the calcium to be diffusible.[1] Assuming that the cerebrospinal fluid is a true protein-free filtrate of blood plasma, the figures obtained by analysis of this fluid indicate a percentage of diffusible calcium that is lower than the above, namely 45 to 55 per cent. The question thus arises whether the cerebrospinal fluid can justly be regarded as a protein-free filtrate of blood plasma. It can hardly be said that this question has been satisfactorily answered. This then leaves in a highly doubtful state all deductions concerning diffusible serum calcium which have been drawn from analyses of cerebrospinal fluid. We will return to this point later.

In animals with tetany due to parathyroid deficiency it is well known that the total serum calcium is decreased, but which

[1] We have adopted the terms diffusible and non-diffusible, proposed by Cameron and Moorhouse, as those least likely to be misleading.

fraction it is that is diminished, the diffusible or non-diffusible, is a matter of dispute. Until quite recently, with no real experimental basis, the general opinion has been that the diffusible calcium is decreased. However, von Meysenbug and McCann (5) and Moritz (6) found that both diffusible and non-diffusible calcium were reduced in about the same ratio. On the other hand, Cruickshank (7) and also Cameron and Moorhouse (2) came to the conclusion that the non-diffusible calcium practically disappeared from the blood serum while the diffusible calcium was but little reduced.

Closely associated with the problem of the state of the serum calcium are the following questions. With what is the non-diffusible calcium of the blood combined? Is there a non-ionic form of calcium present in the blood that is diffusible? Is the diffusible calcium in equilibrium with the non-diffusible and, if so, what is the nature of the equilibrium? Rona and Takahashi (4, 8), in 1911 and 1913, suggested that the non-diffusible calcium was bound to the proteins of the serum and this was tacitly accepted by all later investigators until the publication by Cameron and Moorhouse (2), in 1925, of their paper "Tetany of Parathyroid Deficiency." In this paper Cameron and Moorhouse rejected the above hypothesis and proposed instead that the non-diffusible calcium is bound to some organic substance which may be the parathyroid hormone or, if not, is regulated by the parathyroid hormone. We shall later have occasion to point out that some of the reasoning used by Cameron and Moorhouse to show that the non-diffusible calcium is not combined with the serum proteins is open to question and also we shall bring forward some evidence in support of the view that the non-diffusible calcium is in combination with the serum proteins, although it should be clearly understood that the evidence presented is indirect. As an alternative idea Greenwald (9) has proposed that some of the calcium is bound to an organic substance in a citrate-like combination, the compound being intimately connected with the parathyroid hormone and *probably diffusible*.

The question whether the diffusible calcium is in equilibrium with the non-diffusible portion has not, heretofore, been experimentally investigated. Nevertheless, with no experimental

evidence for or against such an equilibrium, many investigators have assumed that one exists. Thus Cruickshank[2] says:

> "The separation by filtration of the diffusible salts of the blood serum can be effected by subjecting the filter to a pressure of 150 mm. of Hg . . . . but to determine accurately the amounts of a salt in ionic and colloidal form, it is essential not to alter the balance between them by removal from the solvent of the diffusible salt. According to the law of mass action the removal of the ionic calcium would result in a further breakdown of calcium in combination in order to restore the normal balance."

This quotation, we believe, represents the opinion of most investigators in this field. We shall give experimental evidence to show that this is not true and that, if an equilibrium exists at all, the non-diffusible calcium is in equilibrium with only a small fraction of the amount of diffusible calcium that is present in blood serum. These points we will consider in greater detail after we have described our experimental methods and discussed the data presented.

Methods and Experimental Procedure.

The experiments include the determination of the diffusible and non-diffusible serum calcium of a number of young men and women, medical students for the most part, and two determinations each on dogs, rabbits, and calves. The method used is based upon that reported by Moritz (3). Moritz considered his method an ultrafiltration and described it as such. However, as applied to his particular technique, this term cannot be correct. In his earlier experiments Moritz placed 5 cc. of distilled water in contact with the dialyzing sac containing 2 cc. of serum and in his later experiments placed 3 cc. of water outside. In doing this Moritz changed his method from an ultrafiltration, which it is when there is no liquid in contact with the outside of the sac, to a membrane distribution method. It is true that, since the liquid inside the sac is subjected to 150 mm. higher pressure than the liquid outside, there is a passage of liquid to the outside. This liquid probably represents an ultrafiltrate of the blood serum, but in addition to this, there is also a diffusion of the diffusible con-

[2] Cruickshank (7), p. 17.

stituents of the serum to the outside until sufficient time has elapsed for equilibrium to be established between the two sides. Due probably to the mistaken idea of the nature of his method all the results given by Moritz contain an error. The figures given by him for the diffusible calcium are not the *diffusible* calcium but the *diffused* calcium and, to obtain the diffusible calcium, a correction must be added for the amount of diffusible calcium that is still present in the sac at the end of the experiment. To make this correction it is necessary to know the volume of liquid inside the sac at the end of the experiment, but, since Moritz evidently did not measure this, the amount of correction to be added remains in doubt. As they stand, Moritz' diffusible calcium figures are too low and his non-diffusible figures too high. Guided by our experience, we thought it probable that about 1 cc. of filtrate passed from the inside to the outside in the course of an experiment. Making this assumption, we have recalculated his figures for normal rabbits so that they may be compared with the figures we have obtained (Table I). These are tabulated in Table II and are directly comparable to our results in Table I. These recalculated values are in substantial agreement with the two experiments on rabbits carried out by us. Also, since in one set of experiments there were 5 cc. of water outside and in the other only 3 cc., the magnitude of the correction is quite different in the two sets. The good agreement between the recalculated data for the two sets of experiments supports the idea that our applied corrections are nearly correct. It should be pointed out that the error in calculation does not affect the deductions drawn by Moritz concerning the effect on the diffusible and non-diffusible calcium of the blood serum of irradiation, frequent bleeding, ether anesthesia, parathyroidectomy, and injection of parathyroid extract, since the relative relationship of his results remains unchanged.

Preparation of Membranes.—The parlodion solution employed was the same as that described by Moritz (3) though the tubes used for casting and the method of preparing the membranes differed considerably from his. Pyrex glass tubes 7 cm. long, having an inside diameter of 14 mm. at the open end and about 9 mm. at the closed end, were used. It is important that the closed end be well rounded and the glass well annealed and free from frosting, otherwise the membranes may be granulated and fragile

at this point. To obtain membranes of satisfactory permeability and texture the temperature of the room should not be above 20°C. when they are made and the casting tubes must be scrupulously clean and dry. When making a membrane, a tube is filled to the top by letting a narrow stream of the membrane solution flow into the tube, taking care that no air bubbles collect along the sides.

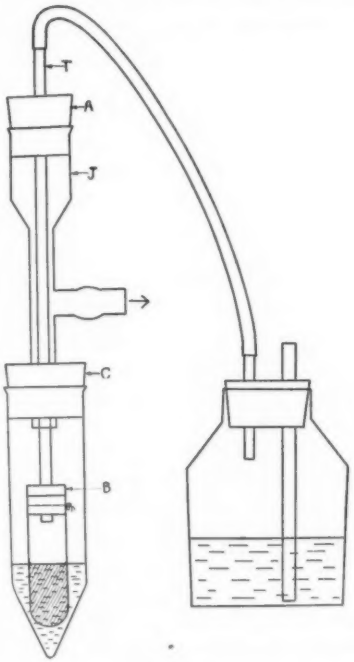

FIG. 1.

The tube is then inverted on the neck of an Erlenmeyer flask and rotated rapidly in a slanting position until drops begin to fall quite slowly. The tube is then refilled, the operation repeated, then placed in a suitable rack, open end down, and allowed to dry for 10 minutes. Such a rack can readily be made by punching holes in a piece of cardboard and fastening it on a test-tube rack. After drying for 10 minutes the tube is plunged into cool distilled

water, the sac removed in the usual manner, and the top cut off so that it is from 5.5 to 6 cm. long. After testing for leaks the sac is dried with filter paper and stored in physiological saline solution (containing a few drops of chloroform to prevent growth of molds) in the refrigerator until required for use. A convenient way to test for leaks is to fill the sac with water and push it on a No. 0 one hole rubber stopper in which a glass tube is inserted. After drying the outside of the sac with filter paper any minute

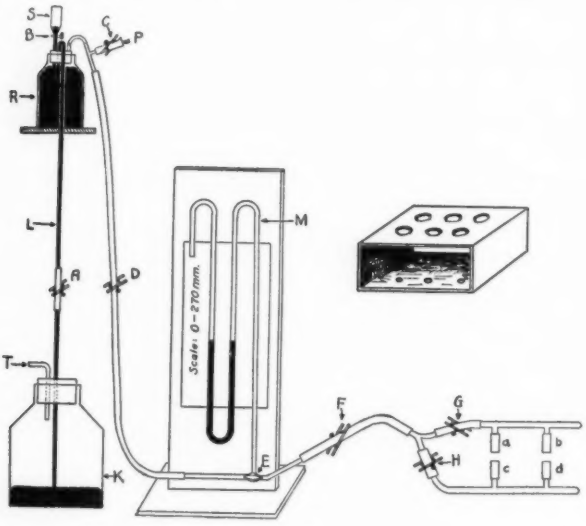

FIG. 2.

leaks can be detected by blowing into the tube while holding the sac securely on the stopper.

Ultrafiltration Procedure.—This differed in a number of respects from that employed by Moritz. Large centrifuge tubes of the type recommended by Clark (10) were used instead of small 15 cc. tubes for the outside vessels. Also the glass tube connecting the parlodion sac with the outside air was connected by a rubber tube to a water wash bottle to keep the air saturated with water vapor and thus prevent evaporation (see Fig. 1). No attempt was made to control the pressure of CO_2 as it has been demonstrated

by von Meysenbug and coworkers (11) and by Neuhausen and Pincus (1) that the CO_2 tension is not an important factor in this connection.[3] A vacuum was applied to the outside of the sac by means of the apparatus shown in Fig. 2 and was obtained by the siphoning action of a 3½ ft. fall of mercury through barometer tubing, the pressure being controlled by means of Screw Clamp *A* and the manometer in the circuit. All glass joints were connected by heavy pressure tubing and then shellacked. Four determinations can be run at one time and, if desired, any one can be shut off by the screw clamps, *a*, *b*, *c*, *d*, as shown in Fig. 2 while the rest are still running. It is important that the glass tube, *T*, Fig. 1, move freely in the rubber stopper, *A*, which fits into the pyrex glass jacket, *J*, without leaking air too rapidly. This is accomplished by boring the hole in *A* a little small, then enlarging with a rat tail file to fit, paraffining the hole, and then rubbing out the paraffin with a glass rod. Moisture on Stopper *A* and Tube *T* should be guarded against as this will cause Tube *T* to stick in Stopper *A*.

To carry out a determination, a parlodion sac is taken from the storage jar (a blunt surgical forceps is convenient for this), carefully dried inside and out with filter paper, and placed in the bottom of a large centrifuge tube (Fig. 1). 3 cc. of serum are then introduced into the sac, drop by drop, keeping the tip of the pipette close to the level of the serum to prevent spattering. The last drop is expelled by breathing gently into the pipette. The sac is then picked up with the forceps and slowly worked on to the stopper, *B* (Fig. 1), care being taken to hold it in a vertical position during the process. When the sac fits snugly on Stopper *B* it is tied with a stout cotton thread, using a square knot. The filtration apparatus is then put together as shown in Fig. 1, placed in one of the holes of the rack illustrated in Fig. 2, and connected to the pressure apparatus at one of the connections *a*, *b*, *c*, or *d*. Before the negative pressure is started each sac should be out of contact with the outside liquid.

To start the vacuum apparatus, the procedure is as follows.

[3] A paper by L. Irving (12) has recently appeared, in which blood serum was found to be supersaturated with respect to calcium carbonate. It was likewise observed that wide variations in CO_2 tension had little effect upon the calcium carbonate equilibrium.

The siphon is formed by exhausting the air in the receiving jar, *K*, with a vacuum pump connected to *T* (Fig. 2). After this has been done the screw clamp at *A* is closed. Before all four filtration vessels are set up and connected to *a*, *b*, *c*, and *d*, Stop-cock *B* and Screw Clamps *A* and *C* should be closed (Screw Clamps *D*, *F*, *G*, and *H* should be open) and the three-way stop-cock, *E*, turned so that the manometer, *M*, is in the circuit. When the glass jackets are all connected, Screw Clamp *A* is opened slightly to permit the building up of negative pressure by the flow of mercury from *R* to *K*. This should be done gradually to prevent sudden changes. When the desired pressure is reached as shown on the manometer scale, Screw Clamp *A* is closed. It was found that, with 20 pounds of mercury in the reservoir, *R*, a negative pressure of 150 mm. could be maintained for over 3 hours without refilling *R*. When the desired pressure is obtained, each sac is gently lowered by means of Tube *T* into the outside liquid until the levels of the inside and outside liquids are the same, and the levels are kept so adjusted during the course of the experiment. When it is necessary to refill *R* with mercury, Screw Clamps *A*, *F*, and *D* are closed, the glass plug, *P*, is removed, and Screw Clamp *C* is opened. The mercury from the receiving jar, *K*, is emptied and poured back into *R* through the separatory funnel, *S*. A small amount of mercury is left in *S* to form an additional seal and Stop-cock *B* is closed. Screw Clamp *C* is then closed and the piece of glass rod inserted at *P*. When the apparatus is set up properly the pressure in the system does not increase more than 5 or 10 mm. while *R* is being refilled with mercury.

At the end of the experiment the sacs are all raised so as to clear the outside liquid, the pressure then allowed to rise gradually to that of the atmosphere, and the glass jackets are then disconnected. Stopper *C* (Fig. 1) is now removed, lifted out, and the sac washed off into the outside liquid with 3 cc. of water, saline, or Ringer's solution, depending upon which of these solutions was used outside the sac. The sac is now carefully removed from Stopper *B* and the serum left in the sac measured into an empty centrifuge tube, using a 1 cc. pipette graduated in 0.05 cc. The sac is then washed out with two successive 3 cc. portions of saline and the washings are added to the serum in the centrifuge tube. The centrifuge tube is now covered with tin-foil and set aside for analysis.

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Determination of Calcium.—The method used for the calcium determinations was that of Clark (10) though a few modifications were introduced. The calcium oxalate was allowed to precipitate overnight as described and, in the morning, the precipitates in the serum, serum plus saline, or the inorganic solutions were thoroughly policed and the sides of the tubes and policeman washed down with N ammonia from a wash bottle. (The original ammonia stock, 10 N, was prepared by running washed ammonia gas into doubly distilled water and stored in a Pyrex bottle.) The tubes were then centrifuged at a moderate speed (1800 R.P.M.) for 7 to 10 minutes. After centrifuging, 10 cc. of the supernatant liquid from the tubes containing serum ultrafiltrate were drawn off without disturbing the precipitate and set aside to be used for running the biuret tests. If a positive test was obtained the whole experiment was discarded. After carefully siphoning off the supernatant liquid the precipitate was thoroughly stirred up with a strong jet of N ammonia from the wash bottle and the sides of the tube washed down with 25 to 30 cc. of N ammonia. The tubes were then centrifuged again for 7 to 10 minutes. At this stage a few crystals of calcium oxalate may often be observed to float on the surface of the ammonia. Usually none can be seen in the serum samples, but in the inorganic solutions the amount floating is often considerable. This is probably a surface tension phenomenon. The supernatant liquid is then siphoned off, taking care not to suck up any of the floating particles. 5 cc. of N sulfuric acid are next drawn up into a pipette and the precipitate stirred up by blowing a fine stream of the acid into the tip of the centrifuge tube. The rest of the sulfuric acid in the pipette is utilized to wash off the crystals of calcium oxalate clinging to the siphon and also in washing down the sides of the centrifuge tube. The centrifuge tube is then covered with tin-foil and set aside to be titrated. While titrating the bath is kept at 81–82°C. and only three tubes are kept in the bath at one time. If more tubes than this are heated at the same time, too much evaporation may take place in the last tubes so that the temperature will fall below 65°C. before the end of the titration. The tin-foil should be removed from each tube before it is placed in the water bath.

There are two possible sources of error in these experiments, besides the errors of analysis. One is the time allowed for the

ultrafiltration and diffusion and the other, and more important, is the nature of the membranes. By varying the time from 3, 4, to 5 hours it was found that 3 hours were usually sufficient if the membranes were good. This is in agreement with the observations of Moritz who found that the calcium in the outer liquid reached a maximum in about 4 hours. However, to be sure the membranes are quite right is more difficult. Usually, when they were made according to our procedure we had no trouble. But, in a few instances, when the membranes were a little too thick or had been used a number of times or had been made on a hot day, they were not sufficiently permeable. Such impermeable membranes may partially hold back calcium even when freely permeable to water. To illustrate this we give in Table III the results of two experiments carried out in membranes made on a hot day as contrasted with experiments carried out on the serum of the same persons when the membranes were satisfactory. We are, therefore, inclined to believe that low values for diffusible calcium are more likely to be in error than high values.

DISCUSSION.

The experimental results are summarized in Table I. Such facts as were thought to be pertinent are given in the table. The volume of water that diffused in the course of each experiment is given so that all the necessary data for the calculations made are present. In the first nine experiments the importance of accurately measuring the volume of serum left in the sac was not appreciated, so the only observation made was that approximately 1 cc. of liquid came through in the course of an experiment. In all the other experiments the volume was measured as accurately as was possible. However, the results of the calculations, using the approximate volumes are in substantial agreement with the results of the other experiments. In Table IV there is given a sample calculation to illustrate the method. Since the calcium in the original serum, in the outside liquid, and the amount remaining in the sac were determined, the calculations were made in two ways. In one the value for the total calcium determined in the original serum and the value for the calcium determined in the outside liquid were used, and the value for the calcium left in the sac was obtained by difference. The results of these calcula-

tions are given under diffusible serum calcium in the column headed "calculated by difference." In the column headed "experimentally determined" the values for the determination of the calcium left in the sac were used in making the calculations. We regard the first method as more reliable, since there is an opportunity for some calcium to be impregnated in the pores of the membrane along with the protein which is adsorbed, as has been shown by Hitchcock (13), and thus not determined in the analysis of the serum inside the sac. It will be noted that in the case of subjects D. S. (both determinations (a) and (b) before and after breakfast), and P. L. (determinations (b) before and after breakfast), there is some doubt that the membranes were entirely permeable to calcium. Both the volume of water and the calcium that diffused were, in these cases, unusually low and the membranes had already been used several times.

If our data are examined to see how they compare with the results of other recent workers in this field, the agreement is found to be gratifying. The agreement between our two determinations on rabbits and the results of Moritz has already been pointed out. Neuhausen and Pincus (1) state that in a few samples of human serum they found the diffusible calcium to be between 45 and 50 per cent by ultrafiltration. This agrees very well with our figures for human serum. However, the results obtained by compensation dialysis for diffusible calcium by von Meysenbug and coworkers (11) and also by Rona and Melli (14) on dogs and humans are considerably higher than our own and this is also true of the results of Cruickshank (7) on normal dogs by compensation dialysis. If now we compare our figures with the results of analysis of cerebrospinal fluid we find here also a satisfactory agreement. The diffusible calcium found by us in the two experiments on dogs agrees well with the figures for the cerebrospinal fluid calcium of normal dogs found by Cameron and Moorhouse (2). The figures for spinal fluid calcium of humans reported by Halverson and Bergeim (15) and by Pincus and Kramer (16) are a little higher than our results for diffusible calcium, averaging about 5.0 mg. per 100 cc. However, it should be pointed out that the average for their total serum calcium figures is also a little higher than the average for our figures, so that the percentage ratio of cerebrospinal fluid calcium to serum calcium

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falls between 45 and 50 per cent, which is in agreement with our figures for percentages of diffusible calcium.

Although there seems to be a satisfactory agreement between the figures for the spinal fluid calcium and the diffusible calcium it cannot as yet be safely concluded that the spinal fluid calcium represents the diffusible calcium of the blood. This is so because it is by no means certain that the cerebrospinal fluid is a protein-free filtrate in equilibrium with the blood plasma. The evidence of chemical analysis does not support this idea. If first the results of analysis on non-electrolytes such as blood sugar, urea, and creatinine be taken into consideration, where there is no such factor as the Donnan equilibrium to complicate matters, it is found that they are usually present in lower concentration in the spinal fluid than in the blood. Myers and Fine (17) found the sugar of the spinal fluid to average 57 per cent of the amount in the blood, creatinine 46 per cent, urea 88 per cent, and uric acid 5 per cent. Egerer-Seham and Nixon (18) give for normal individuals 50 to 70 per cent for sugar, 70 to 170 per cent for creatinine, and 45 to 75 per cent for urea. Thalhimer and Updegraff (19) and others have found the sugar of the spinal fluid to be lower than that of the blood. The general agreement is that the concentration of these constituents in cerebrospinal fluid is not the same as in blood plasma. With respect to the inorganic constituents, however, evidence as to the relationship of spinal fluid to blood plasma is conflicting. Pincus and Kramer (16) from their analyses conclude that the Donnan equilibrium plays an important part in the distribution of electrolytes between the cerebrospinal fluid and the blood. Qualitatively the results for chloride, potassium, calcium, and the reported greater alkalinity of the spinal fluid are in agreement with this idea. However, on this basis no explanation is offered for the fact that sodium and CO_2 (which represents bicarbonate ions) have the same concentration in spinal fluid and blood serum, and the distribution of inorganic phosphorus is in the wrong direction. Moreover, the work of Rona and his collaborators (4, 20), Neuhausen and Pincus (1), and others indicates that the unequal distribution of ions due to the Donnan membrane effect is negligible. Until it has been demonstrated, therefore, by actual comparative analysis that the calcium of the cerebrospinal fluid does represent the diffusible calcium of the blood plasma, we

must rely upon some such method as is described in this paper in the effort to determine the distribution in blood serum of these two forms of calcium.

Level of Diffusible Calcium.—Our results on human subjects show that the diffusible calcium tends to remain more constant than the non-diffusible. This is also true of the results on normal rabbits recalculated from Moritz' data. Graphically the variations in the diffusible and non-diffusible calcium shown by our results on human subjects are illustrated in Fig. 3. Again we wish to call

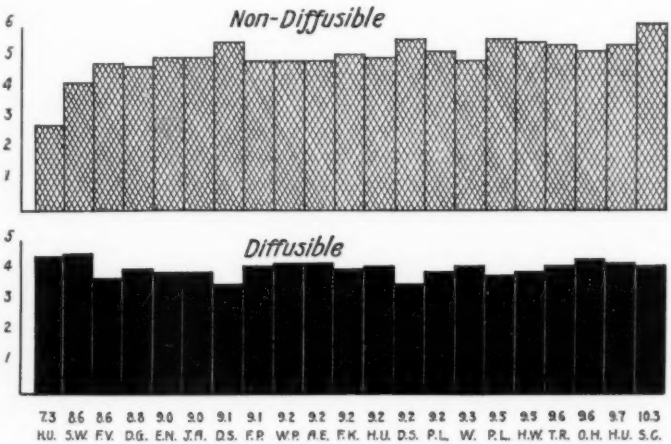

FIG. 3. Graphs constrasting variations in diffusible and non-diffusible calcium of human blood serum.

attention to the fact that in the case of both experiments on subject D. S. (see Table I) there is a reasonable doubt that the membranes were entirely permeable to calcium, as but a very small amount of water came through, especially in experiment (a) after breakfast. This may account for the low diffusible calcium obtained in this particular case, and it will be observed that, were these values omitted in plotting the graphs, the one for the diffusible calcium would assume an even more constant level. It is significant that for the subject with the lowest total serum

calcium, that of H. U. with 7.3 mg. per 100 cc.,[4] and for the one with the highest total serum calcium, S. C. with 10.3 mg. per 100 cc., the diffusible calcium is substantially the same; namely, 4.5 mg. per 100 cc. for H. U. and 4.2 for S. C. This clearly illustrates that the usual procedure for reporting the diffusible calcium in terms of per cent of total serum calcium may be misleading, for, if the level of the diffusible calcium is practically fixed while that of the non-diffusible portion fluctuates within comparatively wide limits, the percentage of diffusible calcium will fluctuate, thus creating the impression that it is undergoing changes when this may not be the case at all. This is again well illustrated in the case of H. U. When her total serum calcium was 7.3 mg. per 100 cc. the percentage of diffusible calcium was 62 per cent, but in the next experiment her total calcium had increased to 9.2 mg. per 100 cc. and the per cent of diffusible calcium was then 46, giving the impression that the diffusible calcium had become greatly reduced, whereas, as a matter of fact, the diffusible calcium in the two cases differs only by 0.3 mg. Our contention that the level of the diffusible calcium is more constant than the level of the non-diffusible is also borne out by the calculation of the usual statistical coefficient of variation. For our human subjects the coefficient of variation is 6.5 per cent for the diffusible and 12.5 per cent for the non-diffusible calcium, while for rabbits the coefficient of variation becomes 10.5 per cent and 34 per cent respectively. The variation of the non-diffusible calcium is, therefore, twice that of the diffusible for our human figures and three times that of the diffusible for the rabbit figures. We are calling attention to this without, at present, placing great weight upon it as a general principle because of the limited data available.

The factors determining the concentration of ionic calcium in a medium of the same hydrogen ion, bicarbonate, and phosphate ion concentration as that of the blood have been theoretically developed, first by Rona and Takahashi (8) and more recently by Kugelmass and Shohl (21) and by Holt, La Mer, and Chown (22). Holt, La Mer, and Chown have shown that the blood serum is supersaturated with respect to tertiary calcium phosphate

[4] Unpublished experiments carried out by G. W. Clark upon prison inmates of San Quentin show variations in serum calcium, for the same individual, which range from 7.3 to 11.0 mg. per 100 cc.

($Ca_3(PO_4)_2$). Whether this is true also with respect to secondary calcium phosphate ($CaHPO_4$) and calcium carbonate[3] remains to be determined experimentally. Calculation of the amount of ionic calcium that ought to be present for saturation by the Rona-Takahashi equation or the more elaborate equation developed by Kugelmass and Shohl:

$$(Ca^{++}) = \frac{7.6 \times 10^{-5}\,[H^+]}{[HCO_3^-]\,[HPO_4^-]}$$

gives about 2 mg. per 100 cc. This is about half the diffusible calcium that our figures indicate for human serum, and only a fourth of that indicated by Moritz' figures for the diffusible calcium of rabbit serum. The fact that the ionic calcium of the serum appears to be such a small fraction of the diffusible calcium has led Greenwald (9) to suspect the existence in the blood of a non-ionic organic complex of calcium that is probably diffusible.

Proof that Diffusible and Non-Diffusible Calcium Are Not in Equilibrium.—If the two forms of calcium were in equilibrium it would naturally follow that, when the concentration of diffusible calcium was lowered, some of the non-diffusible calcium would be converted into the diffusible form to maintain the balance and, conversely, on increasing the concentration of the diffusible calcium, some of the diffusible calcium would be converted into the non-diffusible form. This holds whether the non-diffusible calcium is ionized, as is the case in the Donnan membrane equilibrium, or whether it is held combined in a non-ionic form. In the former case we have an illustration of what Donnan (23) called membrane hydrolysis, and the second is the usual illustration of the mass law relation between a non-ionized compound and its ions. All the evidence at hand indicates that the non-diffusible calcium is present in a non-ionic form, so only the latter illustration of equilibrium need be considered here. This evidence has been accumulated by Rona and Takahashi (4) who employed compensation dialysis, by Cushny (24), and also by Neuhausen and Pincus (1) who employed ultrafiltration. These investigators have found that all the inorganic constituents of the serum are completely diffusible except calcium and probably magnesium. If the non-diffusible calcium were present in an ionized form, such

results would be impossible in view of the condition of the Donnan membrane equilibrium. To the above evidence it should be added that Neuhausen and Marshall (25) found about 20 per cent of the serum calcium to be ionic calcium by the use of a calcium amalgam electrode.

In the series of experiments carried out with no water outside, with 5 cc. of water, physiological saline, or Ringer's solution outside, and the two cases in which, after being in contact for 3 hours with saline, the sacs were transferred to other vessels containing 5 cc. of fresh saline, the diffusible constituents of the serum have been diluted over a fairly wide range, but the amount of diffusible calcium remains practically the same in all. The results obtained with Ringer's solution as contrasted with distilled water are shown in Table I, under subjects F. K., H. U., T. R., and W. Within the limit of accuracy of analysis the diffusible calcium is the same whether the outside solution be Ringer's solution, saline, or distilled water. The experiments, in which the effect of no water outside the sac is contrasted with that of 5 cc. of water outside, were carried out on the sera of subjects S. C., O. H., F. P., J. A., Dog A, Rabbit A, and the two heifers. The experiments carried out on the sera of subjects H. W. and D. G. were run 3 hours with 5 cc. of saline outside and the sacs were then transferred to tubes containing 5 cc. of fresh saline. These two experiments were first attempted with distilled water, but, on transferring to the second tube, water started to flow into the sacs again so, to obviate this, physiological saline was substituted in the set of experiments given in the table. In the experiments carried out with 5 cc. of water, Ringer's solution, or saline outside, the diffusible calcium of the serum is diluted to approximately 35 per cent of its original concentration in the serum although, as our experiments show, the total is not changed. In the experiments in which the sacs were transferred to 5 cc. of fresh saline after 3 hours, the diffusible calcium is diluted to approximately 5 per cent of its original value. At this value it seems likely that we have reached the amount of diffusible calcium with which the non-diffusible is in equilibrium, for, in both these experiments, the figures for the diffusible calcium are a little higher than for the determinations in which the same 5 cc. of saline were used outside of the sac throughout the experiment. However, this point must

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be checked more carefully before a positive assertion to that effect can be made.

To a casual observer there is another interpretation of our figures which may suggest itself; namely, that the two forms of calcium are in equilibrium but that the adjustment is slow and, when this equilibrium is upset, a long time is required to reach it again. (This is the point of view taken by Cameron and Moorhouse (2) and by von Meysenbug and coworkers (11).) However, this can hardly be so, as far as the equilibrium between the diffusible and non-diffusible calcium in the serum uncomplicated by the use of membranes is concerned, for it has been shown by Clark (10) among others that oxalate will precipitate about 90 per cent of the calcium from undiluted serum within half an hour. This indicates that the establishment of equilibrium must occur within this period of time. As a matter of fact the dissociation of the calcium from the non-diffusible calcium complex probably occurs more rapidly than this and the greater part of the time required is utilized by the calcium oxalate crystals in growing to a size large enough to be thrown down by centrifuging. While it may also be argued that, due to slowness in diffusing through the membrane, insufficient time was allowed for equilibrium to be reestablished, this objection seems groundless, since our results and those of Moritz show that the maximum value for diffusible calcium is attained in 3 to 4 hours. In the light of what has been said it would be expected that the experiments with water outside the sac should show higher values for diffusible calcium than the determinations with no water outside, if the level of the diffusible calcium is dependent upon that of the non-diffusible calcium. However, the very close agreement between the experiments on the same sera with no water outside and 5 cc. of water outside leaves but little doubt that, within this range of dilution, there is no equilibrium between the diffusible and non-diffusible calcium.

Is Non-Diffusible Calcium in Combination with Serum Proteins?—This question has been raised by Cameron and Moorhouse (2) who have discarded the theory, which was before this quite generally accepted, that the non-diffusible calcium is combined with the serum proteins. They propose instead that the non-diffusible calcium is in combination with some unknown organic substance that is intimately connected with the functioning of

the parathyroid hormone. The most important evidence which Cameron and Moorhouse brought forward in opposition to this idea of a combination of calcium with the serum proteins was their observation that in parathyroidectomized dogs the total serum calcium was greatly reduced though the calcium of the cerebrospinal fluid was but little lowered. They concluded, however, not that the non-diffusible calcium of the blood had practically disappeared but that it remained behind in the blood clot, since their analyses showed that the plasma of parathyroidectomized dogs contained from 2 to 4 mg. per 100 cc. of calcium more than did the serum of these animals. This is contrary to the findings of Greenwald (9) who, using heparin as an anticoagulant, obtained the same values for the calcium content of the plasma and that of the serum of parathyroidectomized dogs. In normal animals Cameron and Moorhouse (2) conclude that the non-diffusible calcium passes into the serum on clotting which, in their opinion, accounts for the observations that the calcium of the plasma and of the serum are normally equal. While Salvesen and Linder (26) have shown that the level of the serum proteins remains practically unchanged in parathyroidectomized dogs, Cameron and Moorhouse find that the non-diffusible calcium has disappeared from the serum of such animals. It would seem, therefore, from these results of Cameron and Moorhouse taken in conjunction with those of Salvesen and Linder, that the calcium can hardly be bound to the serum proteins. Underlying the reasoning of Cameron and Moorhouse is the assumption that the cerebrospinal fluid is a protein-free filtrate of the blood plasma. For the present, however, for reasons already given, the cerebrospinal fluid calcium can hardly be considered to represent the diffusible calcium of the blood plasma. Moreover, of all the investigators who have studied the diffusible calcium in parathyroidectomized animals, only Cruickshank (7) has come to the same conclusions as Cameron and Moorhouse; *i.e.*, that the non-diffusible calcium practically disappears from the blood serum of such animals. Moritz (6), who has studied the effect of injecting parathyroid extract on the serum calcium of rabbits, found that both diffusible and non-diffusible calcium were increased but that usually the diffusible calcium was increased more than the non-diffusible calcium. This is contrary to the hypothesis of Cameron and Moorhouse that the parathyroid

hormone regulates the level of the organic substance which is combined with the non-diffusible calcium, for, if that were true, it would be expected that parathyroid extract injection would raise the level of the non-diffusible calcium but it would hardly be expected that the level of the diffusible calcium would be raised even more.

One of the two other arguments used by Cameron and Moorhouse (2) seems to be unsound. We quote: ". . . . if the calcium is capable of uniting with plasma proteins functioning as acids, the amount of Ca-protein so formed should bear a ratio to Na-protein of about the same order as that of plasma calcium to plasma sodium (about 1:30)." But, as they point out, it has been shown by Rona, Haurowitz, and Petow (20), Neuhausen and Pincus (1), and H. and C. Tschimber (27) that the sodium is completely or almost completely diffusible and so the combination between calcium and the plasma proteins must be negligible. This argument would only be true if the calcium-protein combination were ionized to about the same extent as the sodium-protein combination. However, it has been shown by Greenberg and Schmidt (28) for the protein casein that, while the combination with sodium gives salt-like solutions which are quite highly ionized, the combination with calcium or other members of the alkaline earth series shows a very low degree of ionization. Thus it was found that between pH 7 and 8 about 70 per cent of the calcium was bound to the casein in a complex, non-ionizable form. If then, a similar combination with calcium is formed by the serum proteins, or any member of the serum proteins, it would seem quite logical that practically all the sodium and only a portion of the calcium should be diffusible. Another finding of Greenberg and Schmidt which is in harmony with the present observation with respect to blood calcium is that the amount of non-ionized casein complex did not continue to increase indefinitely as the amount of calcium was increased, but instead soon reached a maximum level. Of course the existence of such a combination remains to be demonstrated for the serum proteins and the findings for casein can have weight only as an analogy. A start in this direction has been made by Loeb and Nichols (29) who found that euglobulin and pseudoglobulin decreased the diffusibility of calcium through collodion membranes and that the greater the concentration of the

TABLE I.

Subject.	Sex.	Experimental conditions.	Volume of water that diffused.	Total serum calcium per 100 cc.	Diffusible serum calcium per 100 cc.			Non-diffusible serum calcium per 100 cc. Average for subject.	Average diffusible serum calcium. Per cent of total.	State of membranes.
					Experimentally determined.	Calculated by difference.	Average for subject.			
			cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
H. U.*	F.	5 cc. water outside sac.	†							
		5 hrs. at 150 mm. (a)	1‡	7.3	4.1	4.8	4.5	2.8	62	New.
		" "	†							
		(b)	1‡		4.5	4.6				
S. W.*	M.	5 cc. water outside sac.	†							
		5 hrs. at 150 mm. (a)	1‡	8.6	4.9	5.0	4.6	4.2	53	(?)
		" "	†							
		(b)	1‡		4.4	3.3				
W. P.§	M.	5 hrs. at 150 mm. 5 cc. water outside sac. (a)	†							
			1	9.2	4.6	4.2	4.3	4.9	47	(?)
		3 hrs. at 150 mm. " " (b)	†							
			1		4.4	4.2				

F. V.*	M.	5 hrs. at 150 mm.	5 cc. water outside sac. (a) " " (b)	† 1 † 1	8.6	4.1 4.2	3.5 3.5	3.8	4.8	44	(?)
A. E.§	F.	5¼ hrs. at 150 mm.	5 cc. water outside sac. (a) " " (b)	† 1 † 1	9.2	5.0 4.9	3.7 3.6	4.3	4.9	47	(?)
F. K.§	F.	5 hrs. at 150 mm.	5 cc. water pH 6.5 outside sac. (a) 5 cc. Ringer's solution pH 7.9 outside sac. (b)	† 1 † 1	9.2	 4.3	3.5 4.6	4.1	5.1	45	New.
H. U.*	F.	5 hrs. at 150 mm.	5 cc. water pH 6.5 outside sac. (a) 5 cc. Ringer's solution pH 7.9 outside sac. (b)	† 1 † 1	9.2	4.3 4.0	4.4 3.9	4.2	5.0	46	New.
T. R.*	M.	5 hrs. at 150 mm.	5 cc. water pH 6.5 outside sac. (a) 5 cc. Ringer's solution pH 7.9 outside sac. (b)	† 1 † 1	9.6	3.7 4.3	4.0 4.8	4.2	5.4	44	Used for second time.

TABLE I—*Continued.*

Subject.	Sex.	Experimental conditions.	Volume of water that diffused.	Total serum calcium per 100 cc.	Diffusible serum calcium per 100 cc.			Non-diffusible serum calcium per 100 cc. Average for subject.	Average diffusible serum calcium. Per cent of total.	State of membranes.
					Experimentally determined.	Calculated by difference.	Average for subject.			
			cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
W.*	F.	5 hrs. at 150 mm. 5 cc. water pH 6.5 outside sac. (a)	† 1	9.3	3.8	4.1	4.2	4.9	45	Used for second time.
		5 cc. Ringer's solution pH 7.9 outside sac. (b)	† 1		4.3	4.8				
D. S.§	M.	5 hrs. at 150 mm. 5 cc. water outside sac. (a)	0.80	9.1	3.8	3.7	3.6	5.5	40	Used for third time.
		" " (b)	0.70		3.6	3.4				
D. S.*‖	M.	5 hrs. at 150 mm. 5 cc. water outside sac. (a)	0.50	9.2	2.8¶	3.3	3.6	5.6	39	Used for third time.
		" " (b)	0.95		3.8	3.6				
E. N.§	F.	5 hrs. at 150 mm. 5 cc. water outside sac. (a)	0.90	9.0	4.0	4.0	4.0	5.0	44	Used for fourth time.
		3 hrs. at 150 mm. " " (b)	0.75		4.1	4.0				

H. U.*	F.	5 hrs. at 150 mm. 3 hrs. at 150 mm.	5 cc. water outside sac. (a) " " (b)	0.60 0.50	9.7	4.2 4.4	4.3 4.1	4.3	5.4	44	Used for fourth time.
P. L.§	M.	3 hrs. at 150 mm. 2 hrs. at 200 mm. 3 hrs. at 150 mm.	5 cc. water outside sac. (a) " " (b)	0.90 0.30	9.2	4.8 3.2	3.9 2.7¶	4.0	5.2	43	Used for fifth time.
P. L.*‖	M.	3 hrs. at 150 mm. 2 hrs. at 200 mm. 3 hrs. at 150 mm.	5 cc. water outside sac. (a) " " (b)	0.85 0.70	9.5	4.2 4.0	4.2 3.2	3.9	5.6	41	Used for fifth time.
S. C.§	M.	3 hrs. at 150 mm.	No water outside sac. (a) 5 cc. water outside sac. (b)	1.35 1.20	10.3	4.3 4.4	4.0 4.1	4.2	6.1	41	New.
O. H.§	M.	3 hrs. at 150 mm.	No water outside sac. (a) 5 cc. water outside sac. (b)	1.45 1.30	9.6	4.6 4 7	4.2 4 2	4.4	5.2	46	New.

TABLE I—*Continued.*

Subject.	Sex.	Experimental conditions.	Volume of water that diffused.	Total serum calcium per 100 cc.	Diffusible serum calcium per 100 cc.			Non-diffusible serum calcium per 100 cc. Average for subject.	Average diffusible serum calcium. Per cent of total.	State membr of
					Experimentally determined.	Calculated by difference.	Average for subject.			
			cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
F. P.*	F.	No water outside sac. (a)	1.35		4.3	4.3				
		4 hrs. at 150 mm. 5 cc. water outside sac. (b)	1.00	9.1	4.2	3.9	4.2	4.9	46	New.
J. A.§	F.	No water outside sac. (a)	1.25		3.5	4.3				
		4 hrs. at 150 mm. 5 cc. water outside sac. (b)	1.25	9.0	3.9	4.2	4.0	5.0	44	New.
H. W.§	M.	3 hrs. at 150 mm. with 5 cc. saline outside sac, then 4 hrs. 20 min. longer at 150 mm. with 5 cc. fresh saline outside sac. (a)	1.60			4.8¶				
		Calculating 3 hr. period assuming 1.60 cc. water diffused. (a)	1.60			3.9				New.
				9.5			4.0	5.5	42	
		Calculating 3 hr. period assuming 1.55 cc. water diffused. (a)	1.55			4.0				
		5 hrs. at 150 mm. 5 cc. saline outside sac. (b)	1.60			4.6¶				

		5 hrs. at 150 mm.	5 cc. saline outside sac. (b)	1.60			4.6¶				
D. G.*	M.	3 hrs. at 150 mm. with 5 cc. saline outside sac, then 4 hrs. 20 min. longer at 150 mm. with 5 cc. fresh saline outside sac. (a)		1.80			4.9¶				
		Calculated for 3 hr. period assuming 1.80 cc. water diffused. (a)		1.80	8.8		4.1	4.1	4.7	47	New.
		Calculated for 3 hr. period assuming 1.75 cc. water diffused. (a)		1.75			4.1				
		5 hrs. at 150 mm.	5 cc. water outside sac. (b)	1.80			4.6¶				
							Mean.	4.1	5.1		
							Coefficient of variation.	6.5	12.5 per cent.		
Dog A, mongrel.		5 hrs. at 150 mm.	No water outside sac. (a)	1.20	10.6	6.3	6.5	5.9	4.7	56	New.
			5 cc. water outside sac. (b)	1.60		5.4	5.7				
Dog B, bull.		4½ hrs. at 150 mm.	5 cc. water outside sac. (a)	1.45	9.0	5.2	4.6	4.9	4.1	55	New.
			" " (b)	1.40		5.2	4.6				
Rabbit A, small.		5 hrs. at 150 mm.	No water outside sac. (a)	1.60	14.1	8.9	8.9	8.6	5.6	61	New.
			5 cc. water outside sac. (b)	1.15		8.3	8.2				

TABLE I—*Concluded.*

Subject.	Sex.	Experimental conditions.	Volume of water that diffused.	Total serum calcium per 100 cc.	Diffusible serum calcium per 100 cc.			Non-diffusible serum calcium per 100 cc. Average for subject.	Average diffusible serum calcium. Per cent of total.	State of membranes.
					Experimentally determined.	Calculated by difference.	Average for subject.			
			cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	
Rabbit B, large.		4¼ hrs. at 150 mm. 5 cc. water outside sac. (a)	1.80	11.6	7.2	7.1	7.6	4.0	65	New.
		4¼ hrs. at 150 mm. 5 cc. saline outside sac. (b)	1.85		7.8	8.3				
Heifer A.		3 hrs. at 150 mm. No water outside sac. (a)	1.30	8.7	4.8	4.5	4.3	4.4	49	New but rather thick.
		3 hrs. at 150 mm. 5 cc. water outside sac. (b)	1.45		4.1	3.7				
Heifer B.		3 hrs. at 150 mm. No water outside sac. (a)	1.30	9.0	4.7	4.1	4.5	4.5	50	New but rather thick.
		3 hrs. at 150 mm. 5 cc. water outside sac. (b)	1.30		4.8	4.3				

* After breakfast.

† Volume of serum left in sac measured only approximately.

‡ Evaporation not prevented.

§ Before breakfast.

‖ 1 pint of milk at breakfast. Blood taken about 1 hour later. In the case of subjects D.S and P.L. blood was drawn before a hearty breakfast including 1 pint of milk. Blood was again drawn 40 minutes after breakfast in the case of D. S., and 55 minutes later in the case of P. L.

¶ Figure not used in obtaining average.

TABLE II.

Recalculation of Diffusible Calcium in Rabbit Serum from Moritz' Data.

Data taken from first paper.		Total calcium.	Diffusible calcium.		Non-Diffusible calcium.
Table.	Column.		Given by Moritz.	Recalculated.	
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*	*mg. per 100 cc.*
IV	1	13.4	7.9	9.2	4.2
		12.6	7.1	8.2	4.4
	2	15.0	8.1	9.5	5.5
		15.3	8.1	9.5	5.8
	3	15.8	6.5	7.6	8.2
		15.5	6.6	7.8	7.7
	4	12.0	6.4	7.4	4.6
		11.4	6.5	7.6	3.8
V	1	13.1	7.6	8.8	4.3
	2	11.7	6.9	8.1	3.7
	3	12.6	7.8	9.1	3.5
	4	12.1	6.7	8.0	4.1
Second paper.					
I	1	12.6	7.2	9.0	3.6
	9	12.1	6.8	8.5	3.6
II	1	11.0	6.3	7.9	3.1
III	1	12.2	6.5	8.1	4.1
	7	13.1	8.0	10.0	3.1
	11	14.0	7.6	9.5	4.5
	15	12.7	7.3	9.1	3.6
	19	11.8	6.3	7.9	3.9
	23	12.1	7.4	9.2	3.9
	30	12.9	7.3	9.1	3.8
IV	1	13.9	7.8	9.8	4.1
	5	12.9	6.6	8.2	4.7
	8	13.9	6.7	8.4	5.5
	12	11.0	6.3	7.9	3.1
	16	12.7	7.1	8.9	3.8
	20	11.7	6.6	8.2	3.5
Average				8.55	4.37
Coefficient of variation				10.6 per cent.	34.0 per cent.

efore a hearty breakfast including 1 pint of milk. Blood was again drawn 40 minutes after b eakfast in the case of D. S., and 55 minutes later in the case of P. L.

¶ Figure not used in obtaining average.

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TABLE III.

Subject.	Experimental conditions.	Volume of water that diffused.	Total serum calcium per 100 cc.	Diffusible serum calcium per 100 cc.	State of membranes.
		cc.	*mg.*	*mg.*	
H. W.	5 hrs. at 150 mm. 5 cc. water outside sac.	1.20	9.0	2.9	Made on a hot day and partially impermeable to calcium.
D. G.	" " " "	1.55	9.1	2.6	
H. W.	3 hrs. at 150 mm. 5 cc. saline outside sac.	1.60	9.5	4.0	Membranes good and entirely permeable to calcium.
D. G.	" " " "	1.75	8.8	4.1	

TABLE IV.

Sample Calculation.

Subject J. A. 5 cc. H_2O outside sac.

Total Ca = 9.0 mg. per 100 cc.
" " in 3 cc. = 0.270 mg.

	H_2O	Calcium per 3 cc.	Total volume outside.
	cc.	*mg.*	*cc.*
Diffused	1.25	0.1003	6.25
Left in sac	1.75	0.1697	

Calcium in each cc. of liquid outside, $0.1003 \div 6.25 = 0.016$ mg.
Volume left inside sac (corrected for protein,* 0.15 cc.) = 1.60 cc.
Diffusible calcium remaining in sac, $1.60 \times 0.016 = 0.0256$ mg.
" " in 3 cc., $0.1003 + 0.0256 = 0.1259$ mg.
" " " 100 " $0.1259 \times \frac{100}{3} = 4.2$ mg.
Non-diffusible calcium in 100 cc., $9.0 - 4.2 = 4.8$ "

* The correction for the volume occupied by the serum proteins is approximately obtained by assuming the protein concentration to be 6.5 per cent and the density 1.3. Then in 3 cc. of serum the amount of protein present is 0.195 gm. and the volume occupied is 0.15 cc.

proteins the greater the decrease in the diffusibility of the calcium. Furthermore, Csapó and Faubl (30) fractionated the proteins of the serum and found that each fraction carried down with it a certain amount of calcium which was held quite tenaciously. Of the serum proteins, the albumin was found by these authors to be combined with the most calcium.

Additional evidence that the non-diffusible calcium may be in combination with the serum proteins or some of the constituents of the serum proteins is to be found in a comparison of our data with the published figures for the serum proteins. Our results indicate that it is the non-diffusible calcium which maintains more nearly the same level in the serum of all the animals we have studied. The average non-diffusible calcium values taken from our data and the recalculated results of Moritz are in mg. per 100 cc., 5.2 for man, 4.4 for rabbits, 4.4 for dogs, and 4.4 for heifers. If we compare these figures with the analytical figures for the serum proteins as collected by Howe (31), we find for the per cent of total serum proteins for man 7.2, for rabbits 5.5 to 6.0, for dogs 5.5, and for cows 6.7. Roughly then, the mg. of non-diffusible calcium per 100 cc. and the per cent of total serum proteins show a similar trend in the serum of the animals we have studied. This is in general agreement with the findings of Salvesen and Linder (32), who found that the decrease in serum calcium in non-uremic cases of Bright's disease paralleled the decrease in plasma protein. To this it may be added that Clark (33) in the case of rabbits, and Heubner and Rona (34) in the case of cats, found that repeated hemorrhage caused a decrease in the calcium content of the plasma. We are well aware that all the evidence we have submitted has only an indirect bearing on the question of whether the non-diffusible calcium is in combination with the serum proteins. It may well be that only a portion of the components of the serum proteins is concerned in this combination, if such a combination exists. It is quite evident from what has been said that further experimental evidence of a more direct nature is required before this question can be definitely settled.

The results described in this paper have been obtained by us in that department of the Stomatological Research program for which Professor Clark is responsible and we are grateful for the

opportunities provided. We also wish to thank Miss Mary E. Stockle for her valuable technical assistance.

SUMMARY.

1. The method devised by Moritz for the determination of diffusible calcium in blood serum has been improved in a number of respects.

2. A study has been made of the distribution of diffusible and non-diffusible calcium in the blood serum of eighteen normal young men and women, two rabbits, two dogs, and two calves.

3. Attention is called to an error made by Moritz in the calculation of diffusible calcium in the blood serum of rabbits and, by applying a probable correction, the figures given by him for normal rabbits have been recalculated.

4. The results on the serum of human subjects and the recalculated results of Moritz on the serum of rabbits indicate that the concentration of diffusible calcium tends to remain more constant than the concentration of the non-diffusible form.

5. It has been observed that the non-diffusible calcium is not in equilibrium with the total diffusible calcium, though it seems probable that it may be in equilibrium with a small fraction of the total diffusible calcium present in blood serum.

6. Evidence has been brought forward which seems to indicate that the non-diffusible calcium is bound to the serum proteins or some component of the serum proteins in a non-ionized form.

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THE CARBON DIOXIDE TENSION OF CEREBROSPINAL FLUID.

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The CO_2 tension is a necessary factor in determining the acid-base equilibrium of the blood. It has been determined for blood both directly, and indirectly through CO_2 of the alveolar air. The blood, because of oxidation, reduction, forced breathing, and stasis through constriction of the veins, undergoes variations in the CO_2 tension. Does the cerebrospinal fluid which exists in a more isolated system follow the same changes as the blood? It too is a system of which the pH is determined by the ratio of $\frac{BHCO_3}{H_2CO_3}$. It differs essentially from the blood, in that, as it contains no hemoglobin, it has no oxidation-reduction effects; and as it contains little serum albumin or globulin, the effect of protein upon the system is practically eliminated.

The CO_2 tension of the cerebrospinal fluid is known only roughly. Parsons and Shearer (1) obtained data from which they calculated tensions of 48 and 64 mm. of CO_2 in two cases of syphilis of the cord. They used electrometric pH determinations, a plot of the absorption curve, and determination of CO_2 content of fluid drawn without contact with air to calculate the pH of the fluid. K. Meier (2) obtained 35 and 39 mm. on two normal cases. Brock (3), on two normal subjects, obtained values of 24 and 27 mm. The two latter have used the colorimetric pH method at room temperature and the total CO_2 of the fluid as drawn. Since the methods of these papers are not given in sufficient detail to evaluate the errors, accurate analysis of their data is impossible; but it is evident that the variations are so wide among the three as to make a further study necessary.

To determine the H_2CO_3 of cerebrospinal fluid it is necessary

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to know the pH, pK_1', and $NaHCO_3$, for according to the Henderson-Hasselbalch equation

$$pH = pK_1' + \log \frac{BHCO_3}{H_2CO_3} \quad (1)$$

If any three of the four variables are known, the fourth can be calculated. Up to the present we have seen no discussion of the value of pK_1' for cerebrospinal fluid. Two methods are available for evaluating pK_1'—first, determination; second, calculation.

1. Determination of pK_1'.—If a sodium bicarbonate solution is saturated at 38° with a known tension of CO_2, one determines the pH and the total CO_2. The bicarbonate CO_2 plus the known amount of CO_2 dissolved in solution equals the total CO_2. Hence the pK_1' value follows because the other three variables are known (equation (1)).

Cerebrospinal fluid is essentially a solution of $NaHCO_3$ in NaCl. Taking the usual precautions of the Van Slyke technique, cerebrospinal fluid was measured into a tonometer. 1 cc. of Hastings and Sendroy's (4) indicator solution of phenol red for 9 cc. of cerebrospinal fluid was introduced. The amount of CO_2 to give the desired CO_2 tension, 30, 40, 50 mm., was introduced, and the tonometer was rotated at 38° for 15 minutes. The gas phase was then analyzed, taking the pressure into account; the pH at 38° measured by the method of McQuarrie and Shohl (5); and the CO_2 analyzed by the method of Van Slyke and Stadie (6). The tonometer used is shown in Fig. 1. It has the advantage of combining in one piece of apparatus a tonometer and color comparator. No transfer of solution is required, and errors resulting from loss of CO_2 during transfer are eliminated.

A solution containing $NaHCO_3$ 0.025 M with NaCl 0.135 M, making a total ionic strength of 0.160, was also analyzed at varying CO_2 tensions. Our results for pK_1' accord with those recently given by Hastings and Sendroy (7). Their data are more accurate because they measured the pH electrometrically and the values are accurate within 0.005 pH or 0.005 pK. The colorimetric values are accurate only to 0.02 pH or 0.02 pK. Therefore, the results are not given in complete form. Our results can be summarized, however, as follows: Both cerebrospinal fluid and $NaHCO_3$ in NaCl solution of concentration equal to that of

cerebrospinal fluid show the same pK_1' value. This value varied in both cases from 6.10 to 6.17; the average values for the former were 6.13 and for the latter were 6.14, which are within experimental error. This agreement is as good as can be expected from this type of measurement. The conclusion is that within the

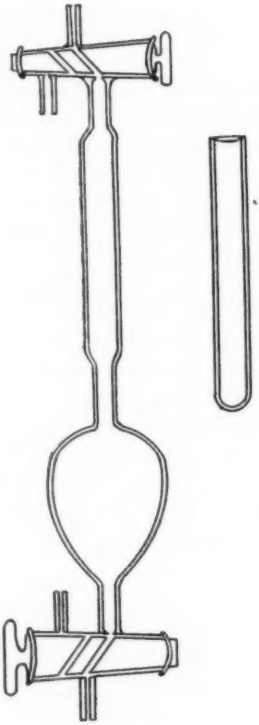

FIG 1.

limits of accuracy of our method cerebrospinal fluid has the same pK_1' value as a solution of NaCl and $NaHCO_3$ of the same composition.

2. Calculation of pK_1'.—The term pK_1' represents $pK_1 + -\log \gamma$ where pK_1 is the value at infinite dilution, and $-\log \gamma$,

the negative log of the activity coefficient at the given conditions. This terminology was introduced into physiologic literature by Warburg, and by Hastings and Sendroy (7) for bicarbonate solutions. The original articles must be consulted for a full description. The familiar equation (1) in terms of activity becomes slightly modified to

$$p\alpha_H = pK_1 - \log \gamma + \log \frac{NaHCO_3}{H_2CO_3} \quad (2)$$

where $p\alpha_H$ represents activity pH, pK_1 is the negative log of K_1, the apparent first dissociation constant of carbonic acid, and $-\log \gamma$ is the negative log of the activity coefficient. The practical advantage of this equation is that $-\log \gamma$ can be calculated according to the concept of ionic strength, introduced by G. N. Lewis. The equation developed by Debye and Hückel for the activity coefficient of a single ion in dilute solution is $-\log \gamma = V \beta \sqrt{\mu}$ where $-\log \gamma$ has the significance just given, β is a universal constant with the value of 0.5, V represents the valence of the ion, and μ, the ionic strength of the system, equals $\frac{1}{2} \Sigma V^2C$, one-half the sum of the products of the square of the valence times the concentration for each ion.

In this case the equation becomes $-\log \gamma = 0.5 \sqrt{\mu}$

To evaluate the $-\log \gamma$ of cerebrospinal fluid it suffices to know the ionic strength of the system. Sufficient data are now at hand to make this calculation, taking the values given by Pincus and Kramer (8) and by Hamilton (9).

	Hamilton. M	Pincus and Kramer. M
Na and K	0.155	0.147
Cl and HCO_3	0.145	0.149
4 × (Ca and Mg)	0.005	0.005
4 × (SO_4 and PO_4)	0.007	0.007
(1) Sum V^2C	0.312	0.306
(2) One-half sum = μ	0.156	0.154
(3) $\sqrt{\mu}$	0.392	0.390
(4) $0.5 \sqrt{\mu} = -\log \gamma$	0.196	0.195

The value of pK_1 given by Hastings and Sendroy (7), in substantial agreement with that of Bray[1] and Walker[1] in the lab-

[1] Unpublished theses submitted to Yale University in partial fulfilment for the degree of Doctor of Philosophy, 1924 and 1925.

oratory of Professor J. Johnston, is $pK_1 = 6.33$. Therefore, for spinal fluid systems $pK_1' = 6.33 - 0.20 = 6.13$. This value has therefore been adopted as the result both of experiment, where the value is 6.13 ± 0.03, and of calculation, where the value is 6.13 ± 0.005.

CO_2 Tension of Cerebrospinal Fluid and Blood Plasma Withdrawn at the Same Time.

Determinations were made of blood plasma and cerebrospinal fluid. The pH of the plasma was determined by Hastings and Sendroy's (4) method, and of the cerebrospinal fluid by the method of McQuarrie and Shohl (5). The CO_2 was determined by the method of Van Slyke and Stadie (6), and the CO_2 tension calculated from equation (3)

$$H_2CO_3 = \frac{1}{R+1} TCO_2 \qquad (3)$$

in which R is the ratio of $NaHCO_3$ to H_2CO_3, and TCO_2 is the total CO_2. The H_2CO_3 can be expressed as mM, as volumes per cent, or taking into account α, the absorption coefficient, it can be expressed as mm. of tension or atmospheres. In the blood the pK_1' value used was 6.10, for the only determination of the pK_1' of plasma calculated from the activity coefficient is that of Van Slyke, Hastings, Murray, and Sendroy (10) for horse plasma of 6.13 and that of Cullen (11) of 6.10 for humans. The α for blood plasma used was 0.541, as has been customary. For spinal fluid, the α was read from a plot of Hastings and Sendroy's (7) newly determined values, and at a concentration of 0.16 M with NaCl and $NaHCO_3$ was taken as 0.535.

Values on normal human beings have not been attainable. However, several cases in which there was no reason to believe that there was any abnormality of the cerebrospinal fluid are included. The results in Table I are given not only in mm. of pCO_2, but also in volumes per cent which can be converted into mM by dividing by 2.24 and have been grouped according to diminishing tensions of CO_2 in the cerebrospinal fluid. These data are not presented as evidence either of the normal values of CO_2 tension or of the pathological variations, for they are not

sufficiently numerous. Nor is it possible to argue the physiological importance of such a system. The main purpose is to show the validity of the method. The applications must be made later. However, certain features stand out prominently. First, though the values of the pH and total CO_2 may be the same respectively in a given cerebrospinal fluid and plasma, the H_2CO_3 is

TABLE I.

	Blood plasma.				Cerebrospinal fluid.			
	pH	Total CO_2.	H_2CO_3*	CO_2 tension.†	pH	Total CO_2.	H_2CO_3*	CO_2 tension.†
		vols. per cent	*vols. per cent*	*mm.*		*vols. per cent*	*vols. per cent*	*mm.*
S. W., tuberculosis..	7.26	58.4	3.75	52.6	7.26	56.8	3.97	56.4
Epilepsy.....	7.37	65.6	3.35	47.0	7.29	57.5	3.77	54.5
H. W., normal.....	7.32	60.0	3.41	47.9	7.32	60.8	3.75	53.2
Dog 1.....	7.34	56.4	3.07	43.1	7.34	62.2	3.68	52.2
J. R., encephalitis.								
Dec. 19.....	7.36	62.2	3.19	44.9	7.28	52.2	3.48	49.5
" 8.....	7.42	60.0	2.80	39.4	7.29	52.2	3.36	48.6
G. E., tuberculosis.								
Dec. 8.....	7.47	43.5	1.78	25.0	7.21	36.2	3.14	44.4
" 15.....	7.47	38.0	1.55	21.8	7.26	39.1	2.72	38.7
" 17.....	7.42	40.6	1.85	26.0	7.27	38.9	2.63	37.3
Retinitis.....	7.39	53.1	2.59	36.4	7.36	54.8	3.02	42.8
Scarlet fever sepsis.	7.35	42.7	2.27	31.9	7.35	37.1	2.13	30.3
Acidosis.....	7.17	14.6	1.14	16.0	7.26	15.2	1.06	15.3

* The pK_1' used for the plasma was 6.10 and for the cerebrospinal fluid 6.13.

† The absorption coefficient used for the plasma was 0.541 and for the cerebrospinal fluid 0.535.

calculated as greater in the cerebrospinal fluid than in blood because of the difference in pK_1' values used. Second, if the α is 0.541 for serum and 0.535 for spinal fluid, the tension of CO_2 must be proportionately greater in the cerebrospinal fluid. Both these factors operate in the same direction so that the calculated CO_2 tension of cerebrospinal fluid should range about 5 to 7 mm. higher than that of blood of the same pH and $NaHCO_3$ content.

The relationship between CO_2 of the blood serum and that of the cerebrospinal fluid has not been determined. Pincus and Kramer

(8) say they are equal. Hamilton (9) says the spinal fluid CO_2 is lower in high or normal values of blood and higher in low values of the blood. Our experience is that either may be higher or lower at any level. It is interesting that the encephalitis cases may show a compensated alkalosis in the blood and a compensated acidosis in the spinal fluid; both result in reduction of CO_2 tension. In the one severe general acidosis, a drop in the cerebrospinal fluid tension corresponds to the drop in tension in the blood.

If one calculates the tension by the method given here, the errors resulting from pH, pK_1', and total CO_2 determinations are

pH	$\pm$ 0.02 at 40 to 60 volumes per cent	= $\pm$2.5 mm.
pK_1'	$\pm$ 0.005	= $\pm$0.5 "
TCO_2	$\pm$ 1 per cent	= $\pm$0.5 "

In calculating the values of the content of H_2CO_3, if the α with which the tension is calculated is known, the tension can be recalculated for any other value. Therefore, in this determination, the error on H_2CO_3 introduced by the uncertainty of the pH is only 0.25 volumes per cent, 5 per cent, or 2.5 mm. The value for pK_1' derived in this analysis is probably accurate $\pm$0.005 and hence more than fulfils the requirements of this determination.

SUMMARY.

1. A method for determining CO_2 tension of the cerebrospinal fluid is presented, based upon the dissociation constant for bicarbonate under the conditions of cerebrospinal fluid. By this method, assuming an uncertainty of 0.02 in the pH, determination of the CO_2 tension may be made with an accuracy of 5 per cent. The value of $pK_1' = 6.13 \pm 0.005$.

2. A compensated acidosis in the spinal fluid may occur simultaneously with a compensated alkalosis in the general circulation.

3. In one case of non-diabetic acidosis a corresponding drop in CO_2 tension took place in the serum and spinal fluid.

4. If the same values of pH and $NaHCO_3$ are found for the blood plasma and cerebrospinal fluid respectively, and if the customary values for the dissociation constant and absorption coefficient of blood plasma are used, the calculated CO_2 tension of the cerebrospinal fluid is greater than that of the blood plasma by 5 to 7 mm.

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ON THE GROWTH-PROMOTING PROPERTY OF IRRADIATED FAT IN THE DIET, OF DIRECT IRRADIATION, AND OF COD LIVER OIL.

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The growth of young rats kept on a diet deficient only in the fat-soluble vitamins (A and D) is enhanced if the animals are irradiated daily by a mercury vapor quartz lamp. Irradiation also induces temporary resumption of growth in rats that have stopped gaining weight on this deficient diet. But that the light cannot entirely replace the fat-soluble, organic growth-promoting factors in the diet is evidenced by the fact that in both cases mentioned above, despite continued daily exposure to the light, the rats eventually cease gaining weight at a point below the normal for their age, and their subsequent history is the same as that of unexposed rats on the same deficient diet (Hume (1), Goldblatt and Soames (2)). These observations have been repeatedly confirmed. It has also been shown that this growth-promoting property can be induced *in vivo* (Goldblatt and Soames (3)); for if rats are kept on a diet deficient in vitamins A and D until they have ceased gaining weight for about 4 weeks, their liver, fed to other rats, does not possess growth-promoting properties; but if, after complete cessation of growth, rats are irradiated for a few weeks by means of a mercury vapor quartz lamp, their livers acquire the property of stimulating for a short while the gain in weight of rats that have ceased growing on a diet deficient in the fat-soluble vitamins. This observation has been confirmed by Steenbock and Black (4), and these investigators and their collaborators (5) have shown in addition that the development of growth-promoting properties can be induced *in vitro* in oils and other substances by exposing them to the rays from a mercury

vapor quartz lamp, carbon arc, and other sources of light rich in ultra-violet rays.

Hume (1), in explanation of the growth-promoting effect of light, suggested that "there is an interaction between vitamin A and light for the growth of rats, but that the action of the light does not produce a photosynthesis of the vitamin." The experiments of Goldblatt and Soames (2) also showed that the growth-promoting effect was not due to synthesis of vitamin A and they suggested that the light in some way economizes the vitamin A still present in the rat and makes it effective to induce growth. Steenbock and Black (4) assumed that the radiant energy of direct light and of irradiated oil, by replacing vitamin D and inducing calcification of bones, also induces growth. But all investigators agree that for continued normal growth the presence of fat-soluble vitamin A (the growth-promoting, antiophthalmic vitamin) is necessary. That the presence of only a small amount of vitamin A in the diet is sufficient to permit normal growth, provided the rats are irradiated daily, was shown by Goldlbatt and Soames (6).

It was considered of interest to compare the growth-promoting power of irradiated fat in the diet, of direct irradiation, and of cod liver oil, and to determine whether irradiated fat in the diet can be used in growth promotion experiments as a substitute for direct irradiation of the animals.

In the case of small animals this would be an advantage, for hooding of the eyes is troublesome and time-consuming, and direct irradiation otherwise induces a conjunctivitis and opacity of the cornea which complicates other eye changes induced by diet, and also probably affects the general condition of the animals.

EXPERIMENTAL.

Since there is no way known at present by which an identical amount of irradiation can be administered directly, by exposure of the animal, and indirectly, by means of irradiated fat in the diet, it was considered best to compare quantities of each which, from previous experiments with the lamp employed, were known to give maximal effects. The comparison was made in two ways.

Part I.—Litters of male albino rats, 28 days old, were first given a diet deficient only in fat-soluble vitamins A and D, (Diet (-A-D)) until they had definitely stopped growing.

Diet (-A-D).

Inactivated* casein	20 gm.
Corn-starch	50 "
Cottonseed oil	15 "
Vegex (for vitamin B)	5 "
Salt mixture (McCollum (7) No. 185)	5 "
Decitrated lemon juice (for vitamin C)	5 cc.
Distilled water	50 "

* Inactivated by the method of Hopkins (8) by heating for 24 hours at 110°C., with a constant inflow of air into the oven.

When, after a varying period on this diet, gain in weight had ceased for not less than 2 weeks, the rats were separated into four groups and treated as follows:

Group I consisted of ten animals which continued to receive only Diet (-A-D).

Group II was composed of twelve rats which received Diet (-A-D), as before, but in addition were exposed daily to the light from a mercury vapor quartz lamp at a distance of 40 cm. from the arc. This group will be referred to in tables and charts as (+I). The lamp[1] employed is a Cooper Hewitt mercury vapor quartz lamp run at 110 volts and 3.5 amperes. The period of irradiation was 5 minutes in the beginning, was rapidly increased in 1 week to 20 minutes daily, an exposure known to be sufficient for maximal growth-promoting effect, and this was continued to the end of the experimental period.

In Group III there were fourteen rats. These received Diet (-A-D), but the cottonseed oil in it was irradiated just before it was added to the diet. This group will be referred to as (+I.C.). The oil, in 150 gm. quantities, in open round dishes 21 cm. in diameter, at a distance of 40 cm. from the arc, was irradiated for 1 hour, during which time it was stirred at frequent intervals. A quantity of oil, sufficient for 1 week's rations, was irradiated at one time.

Group IV had eight rats which continued to receive Diet (-A-D), but in addition were given daily, by mouth, 7 drops (about 150 mg.) of potent cod liver oil, an amount of this oil

[1] This lamp was loaned to us by Professor Miller of Case School of Applied Science, and to him we wish to express our sincerest thanks.

known to be adequate for maximal growth-promoting effect. This group will be referred to in the charts as (+C.L.O.).

Table I and Charts I to IV show that in the four groups the weights of the rats, after they had ceased gaining on Diet (-A-D) alone, varied greatly, but the rats were so chosen for the four groups that the fluctuation of these initial weights is similar. Charts I to IV illustrate the effects of the different treatments which the animals in the four groups received. Table I gives the

TABLE I.

Group I (-A-D).		Group II (+ I.).		Group III (+ I.C.).		Group IV (+C.L.O.).	
Rat No.	Maximum gain.*	Rat No.	Maximum gain.*	Rat No.	Maximum gain.*	Rat No.	Maximum gain.*
	gm.		*gm.*		*gm.*		*gm.*
1486	†	1440	31	1460	33	1429	28
1466	6	1447	45	1461	36	1439	35
1474	3	1448	33	1467	28	1446	72
1483	†	1449	22	1469	21	1459	62
1472	†	1427	43	1444	20	1465	55
1437	10	1438	30	1478	45	1468	66
1457	16	1476	37	1442	48	1484	70
1487	18	1477	42	1426	35	1463	55
1480	†	1485	25	1432	48		
1482	†	1441	28	1450	22		
		1464	5	1433	18		
		1424	9	1481	43		
				1431	5		
				1443	28		

* This represents the maximum gain in weight during a period of 4 weeks following the time when the animals had ceased gaining on Diet (-A-D).

† Indicates that the animal did not gain weight during those 4 weeks and either remained stationary or actually lost weight during that period.

maximum gains which occurred in the four groups during the first 4 weeks after the change from Diet (-A-D) exclusively. The arbitrary period of 4 weeks is chosen because within that period all the rats in Groups II and III had reached their maximal weight and were either maintaining that weight or were already on the decline.

In Group I, only two out of the thirteen animals made an

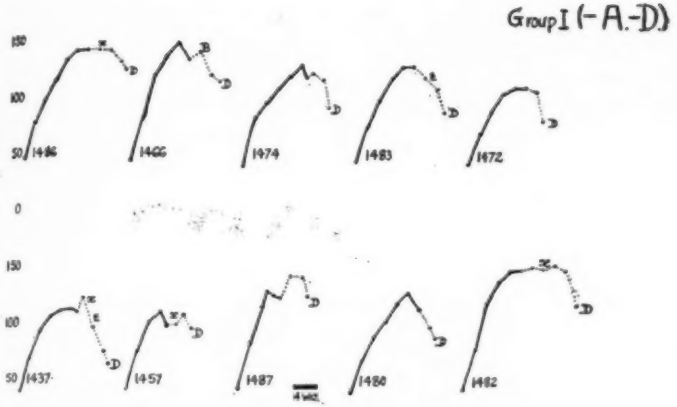

CHART I.

B, Bronchopneumonia. x, Xerophthalmia.
E, Enteritis. D, Died.

Solid line, period on Diet (-A-D) alone until gain in weight had ceased for at least 2 weeks.

Dotted line, period on Diet (-A-D) alone after gain in weight had ceased for at least 2 weeks.

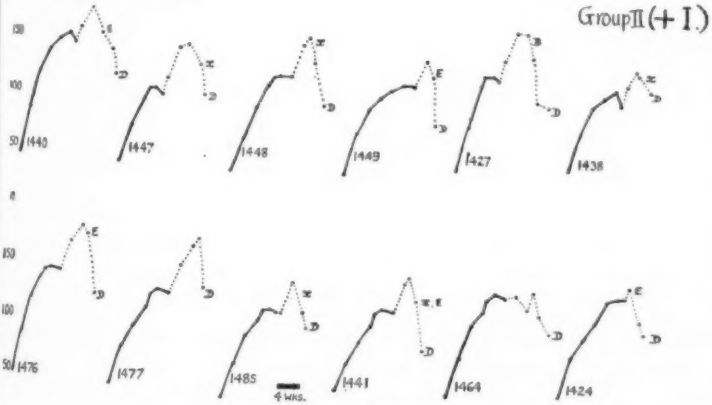

CHART II.

B, Bronchopneumonia. x, Xerophthalmia.
E, Enteritis. D, Died.

Solid line, period on Diet (-A-D) alone until gain in weight had ceased for at least 2 weeks.

Dotted line, period on Diet (-A-D) + daily irradiation of the animals by a mercury vapor quartz lamp.

appreciable gain which, however, was much smaller than the mean gain in the other groups. Of the remainder, three gained a few gm. and eight lost weight. Some developed xerophthalmia, and in some, bronchopneumonia or enteritis, or both, occurred as

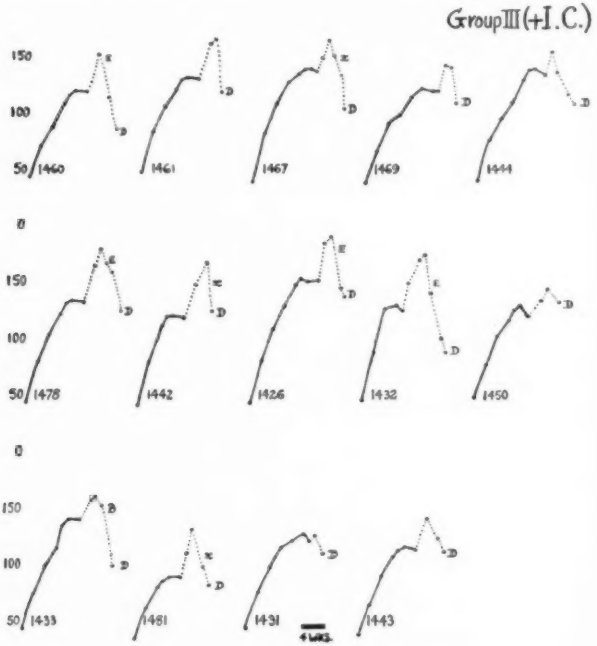

CHART III.

B, Bronchopneumonia. x, Xerophthalmia.
E, Enteritis. D, Died.

Solid line, period on Diet (-A-D) alone until gain in weight had ceased for at least 2 weeks.

Dotted line, period on Diet (-A-D) + irradiated cottonseed oil, after gain in weight had ceased on (-A-D) alone.

complications of the vitamin deficiency, and all finally died, severe loss of weight usually preceding death.

In Groups II and III, the maximum gains varied, but the gains were of similar magnitude in both (see Table I). All the rats in

these two groups had ceased gaining weight after 4 weeks, and their subsequent history was practically the same as that of Group I (see Charts II and III). In many instances the loss of weight was greater and more abrupt than that which occurred in the case of Group I rats.

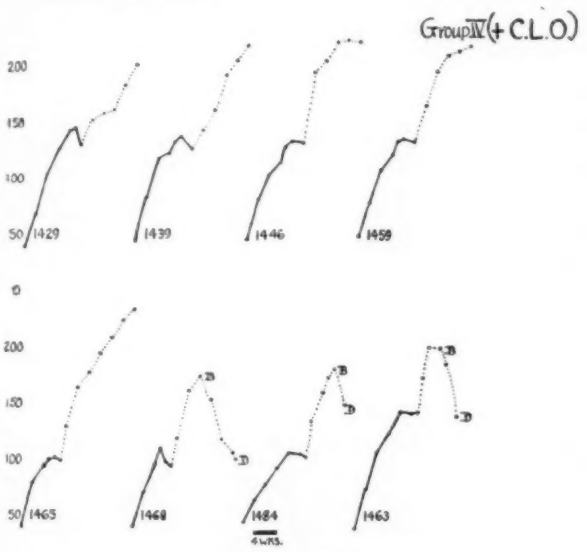

CHART IV.
B, Bronchopneumonia.
D, Died.

Solid line, period on Diet (-A-D) alone until gain in weight had ceased for at least 2 weeks.

Dotted line, period on same diet + 150 mg. of cod liver oil daily after gain in weight on Diet (-A-D) alone had ceased.

In Group IV the gains in weight in 4 weeks were also variable but, in general, they were much greater than those in Groups II and III. Besides, most of the animals in Group IV continued to gain weight for many weeks following the arbitrary period of 4 weeks and appeared to be normal. Bronchopneumonia developed in three rats of this group and finally led to their death. This, in our experience, is an exceptional occurrence in rats on the complete diet and must be regarded as accidental.

The experiment was continued until all the animals in Groups I, II, and III had died. Table II shows that the total length of

TABLE II.

Group I (-A-D).		Group II (+ I.).		Group III (+ I.C.).		Group IV (+ C.L.O.).	
Rat No.	Total length of life.	Rat No.	Total length of life.	Rat No.	Total length of life.	Rat No.	Total length of life.
	wks.		*wks.*		*wks.*		*wks.*
1486	23	1440	23	1460	19	1429	*
1466	19	1447	18	1461	18	1439	*
1474	19	1448	20	1467	20	1446	*
1483	19	1449	20	1469	20	1459	*
1472	17	1427	20	1444	21	1465	*
1437	21	1438	18	1478	20	1468	22
1457	15	1476	20	1442	17	1484	22
1487	16	1477	20	1426	20	1463	22
1480	19	1485	18	1432	19		
1482	24	1441	19	1450	19		
		1464	22	1433	20		
		1424	18	1481	17		
				1431	17		
				1443	19		

* Indicates that these animals were still alive at the end of the experiment.

TABLE III.

Group I (-A-D).		Group II (+ I.).		Group III (+ I.C.).		Group IV (+ C.L.O.).	
Rat No.	Maximum gain.*	Rat No.	Maximum gain.*	Rat No.	Maximum gain.*	Rat No.	Maximum gain.*
	gm.		*gm.*		*gm.*		*gm.*
1512	52	1515	86	1518	95	1521	146
1513	33	1516	103	1519	96	1522	132
1514	44	1517	98	1520	89	1523	118
1524	51	1527	134	1530	150	1533	180
1525	76	1528	110	1531	74	1534	178
1526	24	1529	95	1532	79	1535	213

* This represents the greatest gain in weight made during the entire period of the experiment.

life of the rats in the four groups varied greatly but that there were no notable differences between Groups I, II, and III. Of the eight rats in Group IV, five were still living and gaining weight at

the end of the experimental period, which lasted 20 weeks. The three that died of bronchopneumonia were 22½ weeks old at the time of death so that even those three lived as long as the maximum in Groups II and III (see Table II).

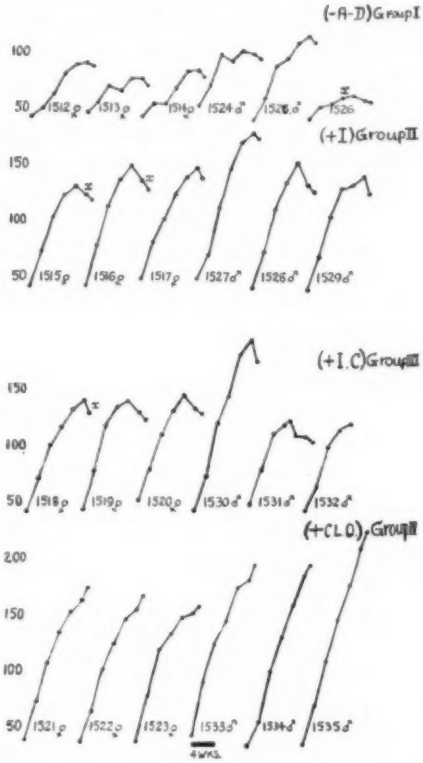

CHART V.

x, Xerophthalmia.

Part II.—The second method of comparing the growth-promoting property of irradiated fat, of direct irradiation, and of cod liver oil was as follows:

Litters of rats 28 days old were divided into four groups, each

containing three males and three females. The groups corresponded to those in Part I of this communication, the only difference being that the rats were separated into these groups at the beginning of the experimental period without being placed for a preliminary period exclusively on Diet (-A-D).

Table III shows the maximum gains in weight during the entire period of the experiment, which was interrupted at the end of 11 weeks, when all the rats in Groups I, II, and III had stopped gaining. That the animals used in this experiment did not have as great a store of the fat-soluble vitamins as those used in Part I, is evidenced by the fact that those in Group I (on Diet (-A-D) exclusively) did not grow as well as the ones in the corresponding group of Part I. The maximum gains by the rats in the four groups varied greatly, but Table III and Chart V show that the fluctuations in Groups II and III are of about the same order, that the maximum gains in these two groups are much greater than those in Group I, and much less than those in Group IV. The animals in Groups II and III grew more rapidly and continued to grow for a longer period than those in Group I and during the first 6 weeks of the experimental period grew almost as well as those in Group IV.

SUMMARY.

By two methods an attempt has been made to compare the growth-promoting power of irradiated fat in the diet, of direct irradiation, and of cod liver oil.

Rats which received a diet deficient in Vitamins A and D (-A-D) until they had ceased gaining weight for a period of about 3 weeks, when exposed daily to the light from a mercury vapor quartz lamp (+I) or given irradiated fat in the diet, resumed moderate gain in weight for a period of from 2 to 4 weeks. The maximum gains during 4 weeks were practically of the same order in both groups, but much less than those of another group which received cod liver oil (+C. L. O.).

Rats which, from the beginning of the experimental period, received Diet (-A-D) and, in addition, were either irradiated directly, or given Diet (-A-D) which contained irradiated cottonseed oil, reached similar weights which were much greater than

those of rats on Diet (-A-D) exclusively, but not as great as those of rats on Diet (-A-D) that received daily about 150 mg. of cod liver oil.

CONCLUSIONS.

Irradiated fat in the diet and direct irradiation possess to about the same degree the power to promote gain in weight of rats on a diet deficient in both fat-soluble vitamins (A and D), but this power is less than that of cod liver oil which, by reason of the fact that it is rich in vitamins A and D, possesses the growth-promoting property to a far greater degree than either direct irradiation or irradiated oil.

Thus irradiated oil in the diet can be used in growth promotion experiments as a substitute for direct irradiation of animals, but neither source of radiant energy can act as a complete substitute for cod liver oil unless fat-soluble A is also administered.

Radiant energy, administered directly or indirectly, although it prolongs and enhances growth, does not prolong the life of rats on a diet deficient in vitamins A and D and does not prevent them from developing xerophthalmia.

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XUM

THE EFFECT ON THE KIDNEY OF THE LONG CONTINUED ADMINISTRATION OF DIETS CONTAINING AN EXCESS OF CERTAIN FOOD ELEMENTS.

I. EXCESS OF PROTEIN AND CYSTINE.*

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During the last few years several investigators have made an experimental study of the effects on growth and nutrition of the administration of a relative excess of certain food elements. Under uncontrolled conditions, of course, such experiments have been made on a vast scale over long periods of time. In arctic regions, for as long as man has lived there, for so long has he had to exist on a diet which contains a high concentration of protein and a minimal amount of carbohydrate, while in certain tropical regions his food for an unknown number of generations has contained a relatively small amount of protein. Opinions have been expressed as to the influence of such differences in diet on the incidence of Bright's disease and of arteriosclerosis, and these views, though they seem to have little basis in actually observed fact, are largely responsible for the present interest in the effect of high protein diets on the kidney. But the experimental work which has been reported has left the question undecided, for while some investigators find that pathological effects are produced in the kidney by diets high in protein, others observe enlargement without any abnormality while still others fail to see that any effect at all is produced. It is evident that further data are needed for the solution of the problem and in this paper we give the results obtained with a diet which contained an excess

* This work was aided by the Wellington Gregg Fund for the investigation of Bright's disease.

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of protein. We have also carried out experiments in which cystine was given in excess, because it has been suggested that the renal lesions which some have found to follow an excess of protein are due to certain amino acids, of which cystine is one, and because some evidence has been presented to show that cystine alone may damage the kidneys.

To obtain clear and unambiguous results in experiments of this nature certain prerequisites must be met. First the period of observation must be extended to cover a considerable part of the total life span of the subjects of the experiment. Before this work was commenced we had found that even pronounced excesses of various food elements had no pathological effect on the kidney of the rat over periods of 54 days, a time which corresponds to nearly 4½ years in the life of a man. We have continued to use white rats in these experiments but we have extended the period of observation to a year which according to Donaldson's estimate is equivalent to 30 years of a man's life. In the second place it is important that the addition of the excess element shall be the only difference between the control and experimental animals. Our control and experimental groups were made as nearly identical as possible. At the beginning they were all exactly 30 days old and of course were of the same sex. They were selected from a large stock originally derived from Dr. Slonaker's colony which had been reared in this laboratory through many generations on a constant diet and under very uniform external conditions. In each control and experimental group the individuals were so chosen that the range of variation in body weight was nearly the same while the average weight approximated the normal average stock weight at that age. Throughout the experimental period the animals were kept side by side in the same constant temperature room into which no light came which had not first passed through glass. They were all housed in cages of the Slonaker type (1), fed in the same way, and handled for weighing at the same time. But the most essential condition of all is that the control and experimental diets shall both be amply adequate for growth and maintenance and as closely comparable as is possible except for the experimental variable. It is of course impossible to add an excess of protein to the control diet without diminishing to a proportionate degree some of its other constituents, and there

is here a danger that the concentration of one or another essential element may be so lowered that the high protein diet becomes defective. In that case any effect produced might be ascribed to the excess of protein although it might really be related to the unsuspected deficiency. We have accordingly taken special pains to make sure that our high protein diet contained in abundance all necessary food factors.

The control diet which is the basis of all our experimental diets is given in detail in Table I.

TABLE I.

*Control Diet.**

Ingredient.	Per cent.	Protein.	Carbohydrate.	Fat.	Water.	Roughage.	Salts.
Corn-starch (cooked, dried, and powdered)	44.0	0.0	40.4	0.0	3.6	0.0	0.0
Casein (powdered)	16.0	14.4	0.0	0.0	1.5	0.0	0.1
Lard	14.0	0.0	0.0	14.0	0.0	0.0	0.0
Cod liver oil	10.0	0.0	0.0	10.0	0.0	0.0	0.0
Salt mixture (Osborne and Mendel)	4.0	0.0	0.0	0.0	0.0	0.0	4.0
Yeast (dried and powdered) Harris	10.0	2.6	3.7	0.6	0.4	0.2	2.5
Alfalfa (dried and powdered)	2.0	0.3	0.7	0.1	0.2	0.6	0.1

Element.	Per cent of diet.	Per cent of calories.
Protein	17.3	14.6
Carbohydrate	44.8	37.8
Fat	24.7	47.6
Water	5.7	
Roughage	0.8	
Salts	6.7	

* 1 gm. = 4.84 calories.

The high protein diet is given in Table II.

It will be noted that the high protein diet is identical with the control diet except that all of the corn-starch and lard has been replaced by casein. It still contains the same concentration of salt mixture and alfalfa and the same 10 per cent of both cod liver oil and dried yeast, but there is 69.5 per cent of protein as compared with 17.3 per cent in the control diet.

There was no difficulty in making the cystine diet almost identi-

cal with the control diet. It was prepared by mixing 1 gm. of cystine with 99 gm. of the control diet.

We knew that our control diet was adequate because during life the growth curve was better than the average and after death no constant abnormality was found in any of the organs or tissues. If now it were found that the high protein and cystine diets could also support growth and maintenance it might be supposed that this fact would demonstrate their adequacy, so that any organ defect which might occur could not well be thought of as possibly arising from dietary deficiency. This assumption has of course

TABLE II.

*High Protein Diet.**

Ingredient.	Per cent.	Protein.	Carbohydrate.	Fat.	Water.	Roughage.	Salts.
Casein (powdered)	74.0	66.6	0.0	0.0	6.9	0.0	0.5
Cod liver oil	10.0	0.0	0.0	10.0	0.0	0 0	0.0
Salt mixture (Osborne and Mendel)	4.0	0.0	0.0	0.0	0.0	0 0	0.0
Yeast (Harris)	10.0	2.6	3.7	0.6	0.4	0.2	2.5
Alfalfa (dried and powdered)	2.0	0.3	0.7	0.1	0.2	0.6	0.1

Element.	Per cent of diet.	Per cent of calories.
Protein	69.5	71.2
Carbohydrate	4.4	3.8
Fat	10.7	25.0
Water	7.5	
Roughage	0.8	
Salts	7.1	

* 1 gm. = 4.03 calories.

often enough been made in feeding experiments, but it is none the less unwarranted. For it is always possible that a food which contains an excess of any one element may thereby be rendered so unsuitable that it will not be eaten in sufficient quantity and that in certain organs deficiency effects may then appear although the body weight remains uninfluenced. The final exclusion of food deficiency as a possible factor can be accomplished only by measurements of the food consumption. In our experiments this was made a relatively simple matter by the construction of

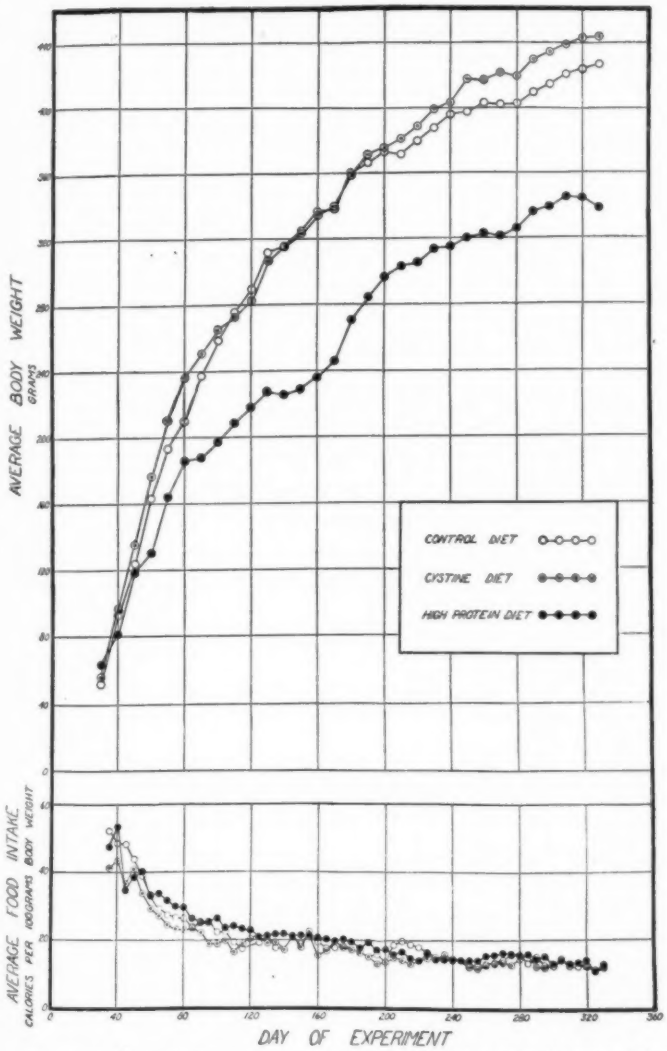

FIG. 1. Average body weight and food consumption of male rats of the first series of experiments (330 days). Control diet = ○, cystine diet = ⊙, high protein diet = ●.

food boxes from which the rats were able to remove only the food which they had swallowed. The food mixtures were so oily that there was no loss of weight by evaporation so it was only necessary to weigh the box after an excess of food had been introduced to

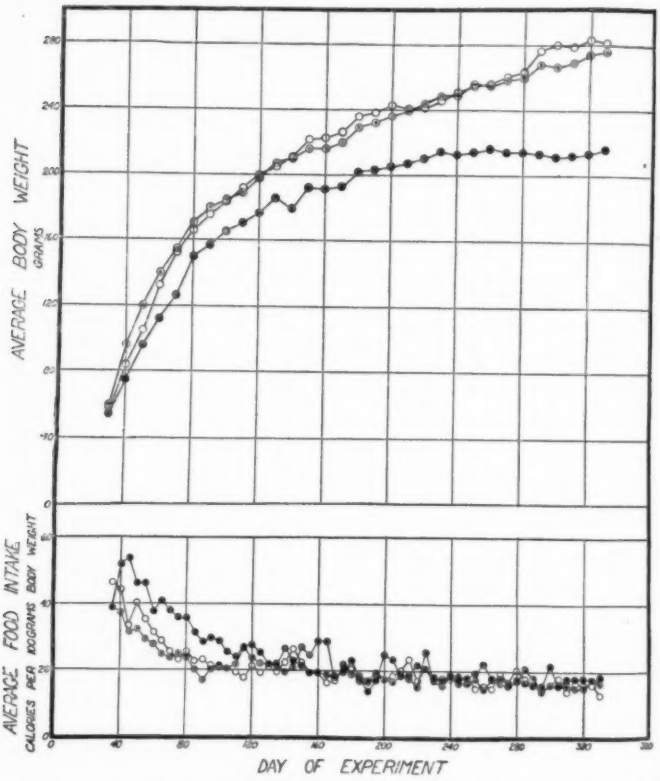

FIG. 2. Average body weight and food consumption of female rats of the first series of experiments (330 days). Control diet = ○, cystine diet = ⊙, high protein diet = ●.

determine at a later date from the decrease in weight how much food had been eaten. The food intake of each group of six rats was thus measured every 5 days and the body weight of each

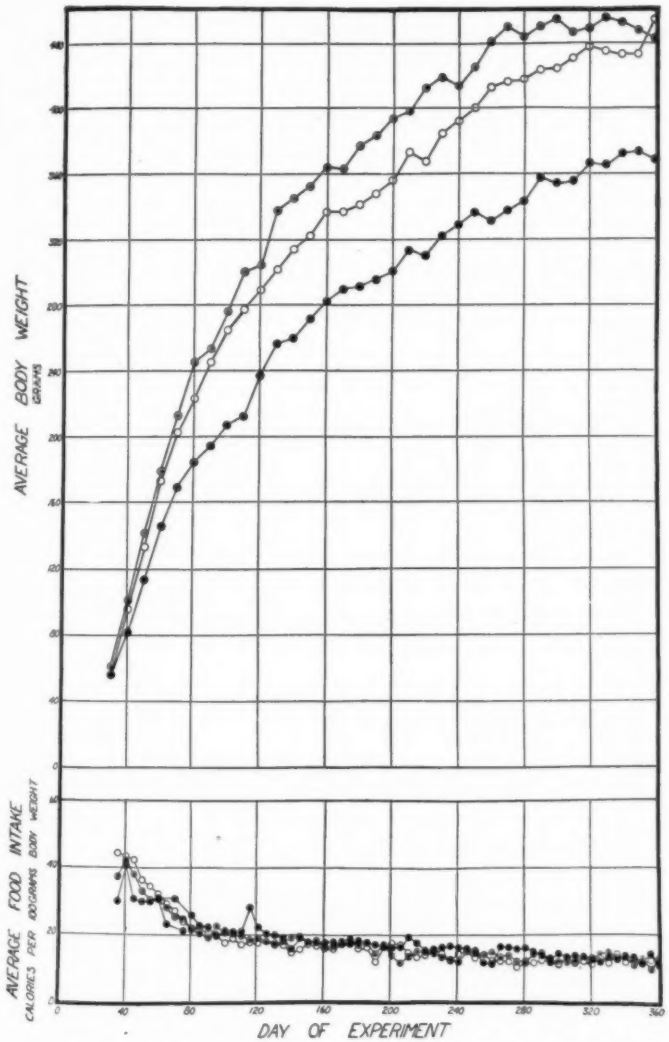

FIG. 3. Average body weight and food consumption of male rats of the second series of experiments (360 days). Control diet = ○, cystine diet = ⊙, high protein diet = ●.

individual rat was noted every 10 days. From these data the graphs of the average food consumption and body weight for each experiment have been constructed. Figs. 1 and 2 are the results on the first series of experiments, male and female groups

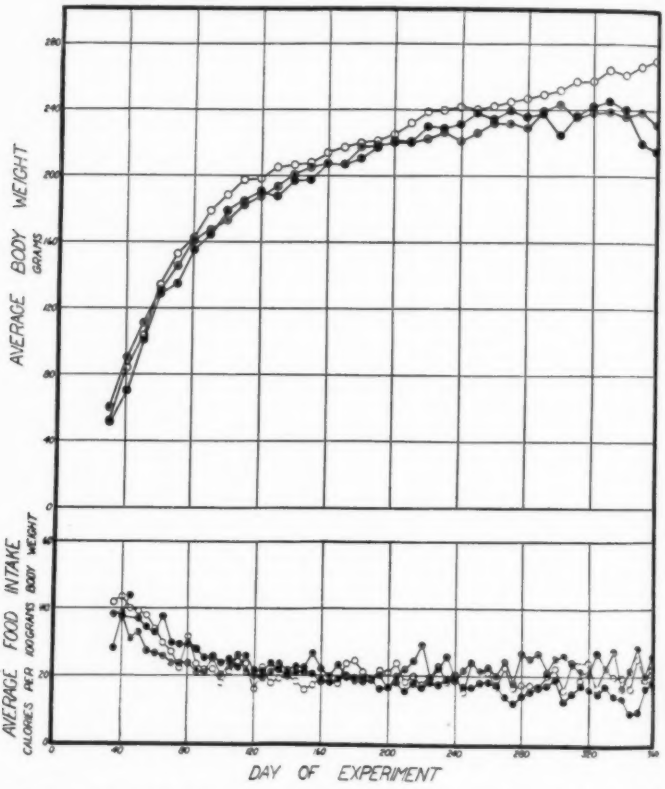

FIG. 4. Average body weight and food consumption of female rats of the second series of experiments (360 days). Control diet = ○, cystine diet = ⊙, high protein diet = ●.

which lived on the diets from 30 days of age to 330 days, and Figs. 3 and 4 are from the second series, male and female groups which were not killed until they were 360 days old.

The outstanding feature in these graphs is the failure of the rats on the high protein diet to attain to the weight of the controls. With the exception of the female group shown in Fig. 4 there is from the beginning a failure to grow as quickly as the controls and as time goes on the weight differences widen. The food consumption curves show that in general the high protein groups ate slightly more calories per 100 gm. of body weight than the controls and they therefore took appreciably more vitamins and minerals since their food had a caloric value of only 4 calories per gm. whereas the control diet had a value of 4.8 calories per gm. It is therefore certain that the lower body weight of the high protein groups was not due to an insufficient supply of any essential food.

TABLE III.

Live Body Weights in Gm. at 330 Days of Age.

	Donaldson's average.	Controls.		Cystine.		High protein.	
		Series 1.	Series 2.	Series 1.	Series 2.	Series 1.	Series 2.
Male.....	278	426	435	444	456	336	366
Female.....	(230)*	282	265	277	240	217	247

* Some of the rats from which Donaldson derived this average had borne litters. Such animals are generally appreciably heavier than rats of the same age which have never been pregnant, as was the case with our animals.

The body weight of the rats on the high protein diets was defective as compared with our controls but it was greater than the standard normal values given by Donaldson (2). The body weights on the 330th day of age are compared with Donaldson's in Table III. There was thus no growth inhibition, only a failure to reach the quite unusual weights of our control and cystine rats.

We had expected to follow the urinary changes throughout the whole course of the experiments, but it was not until more than half of their total duration had elapsed that we succeeded in devising fairly satisfactory methods for a quantitative measure of the changes in the rate of excretion of the protein and the formed elements in the urine. For the collection of urine each animal was placed in a small round wire cage set over a glass funnel. At the beginning and end of a 12 hour period the bladder was emptied by making the rats inhale a little ether. This pro-

cedure is invariably followed by urination. At the end of the period the rat was held over a centrifuge tube while the ether was given, and in this manner clean urine was obtained for microscopical examination. The volume thus directly collected averaged nearly 20 per cent of the total amount in 12 hours and so no serious error was introduced by calculating from the number of cells counted in this portion what might have been expected if the whole volume had been sampled. The technique of counting has recently been described (3). The method of Shevky and Stafford (4) was used for the determination of protein but it was found that irregular results were obtained unless the urine was diluted to five times its volume with water. Since the concentration of protein in rat urine is sometimes quite low we had to have special tubes with fine graduations constructed. By

TABLE IV.

Averages of 12 Hour Rates of Excretion in Urine from Series 1 and 2 Expressed as Rates per Gm. of Renal Tissue.

	Male.			Female.		
	Control.	Cystine.	High protein.	Control.	Cystine.	High protein.
Volume, *cc.*	1.2	1.1	2.0	1.3	1.3	1.8
Protein, *mg.*	2.2	2.9	4.1	0.7	0.65	0.96
White and epithelial cells	24,000	30,000	21,200	30,000	27,000	32,000

these means data were collected which, though negative from the point of view of the detection of renal abnormalities, have an interest of their own. The average results as rates per gm. of renal tissue are given in Table IV.

The number of white blood cells and of epithelial cells shows no significant variation in any of the experiments. The greater volume of urine from the rats on the high protein diet is only what might have been anticipated. But the higher rate of protein excretion in the males than in the females was unexpected and as yet no adequate cause has been found for the difference. It will be noted also that the average rate of excretion is appreciably higher in both males and females in the high protein than in the control or cystine rats. The average individual rates from all the groups are charted in Fig. 5.

These graphs reveal the fact that the average increase in the rate of protein excretion by the rats on the high protein diet arises from a temporary marked increase and is not due to a constantly maintained higher level of excretion nor to any steadily

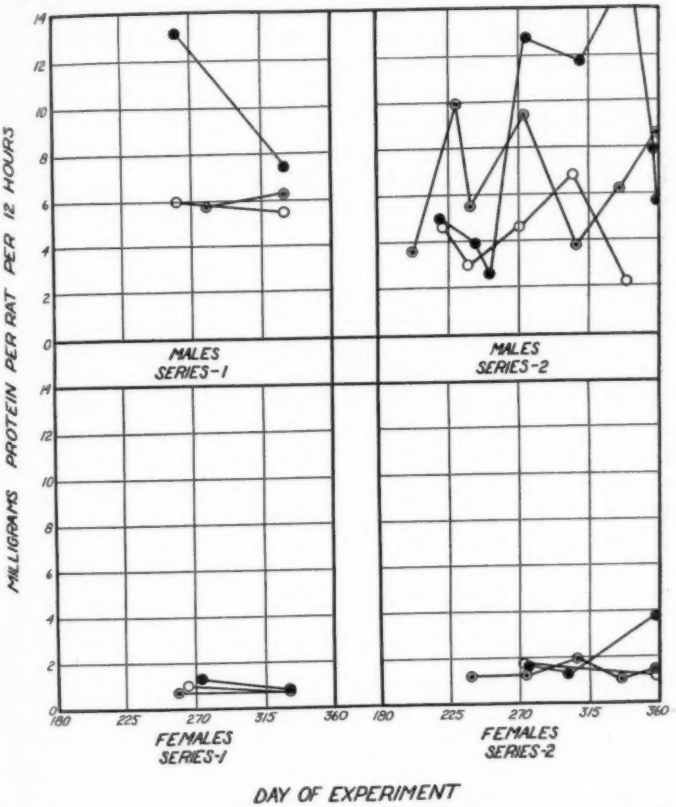

FIG. 5. The rate of protein excretion in the urine in mg. per rat per 12 hours. Control diet = ○, cystine diet = ⊙, high protein diet = ●.

augmenting rise as the duration of the experiment is extended. On this account its meaning is uncertain and will probably remain obscure until we learn something of the general significance of the excretion of protein by all rats.

Red blood cells were often present in considerable numbers in the urine of the control rats. In 78 control urines there were 2 in which there was macroscopic blood and lesser degrees of hematuria recognizable only on microscopical examination were frequently but quite irregularly observed. In the cystine groups 4 urines out of 84 were grossly bloody and in the high protein experiments in 90 urines examined 3 were found to be red or brown with blood. It seems likely that at one time or another red blood cells were present in the urine of every male rat under observation. In the females they were definitely less often seen. Although the total amount of blood excreted was probably somewhat greater in the cystine and high protein rats than in the controls, the difference was certainly slight.

An occasional hyaline cast was found at one time or another in the urine collected from nearly all of the experiments. They were no more frequent in the high protein than in the control or cystine groups. In the rat as in man a small number of casts is constantly being excreted, so that it is not the presence of casts but an increase in their number which indicates an abnormality of the kidney. Since the concentration and reaction of the urines we examined were favorable to the preservation of casts we regard our negative findings as strong evidence against the existence of any active renal lesions of the types met with in Bright's disease.

The single instance of a positive finding remains to be described. It occurred in a male in the first series on the cystine diet. A marked increase in the rate of protein excretion and the presence of numerous casts was noted at the first examination at the 258th day of age. At the end of the experiment the concentration of protein in the urine was over 2 per cent and 51 mg. were excreted in 12 hours. The number of casts was calculated as 82,000 per 12 hours. Of these 1 per cent was epithelial, 0.5 per cent waxy, and the remainder was hyaline casts sprinkled over with fat droplets. The number of epithelial cells and of white blood cells was greatly increased and present in greater number than the red blood cells. It was estimated that in the 12 hour urine there were 1,240,000 epithelial and white blood cells and 330,000 red blood cells. In a human urine such a sediment is typical of an active degenerative renal lesion. This rat had grown rapidly

up to its 80th day but had lagged somewhat thereafter though it never fell appreciably beyond the group variation and in the end weighed only 5 gm. less than another rat in the same experiment. The blood urea concentration on the 330th day was 68.2 mg. per 100 cc. while the average for the rest of the group was 27.5 mg.

At the end of the experiments the animals were anesthetized and while the heart was still beating blood was collected from the abdominal aorta. The blood urea concentration was determined

TABLE V.

Average Blood Urea Concentrations. Mg. per 100 Cc.

	Male.			Female.		
	Control.	Cystine.	High protein.	Control.	Cystine.	High protein.
Series 1.....	30 2	27.5	(34.5)	33.5	41.7	62.9
" 2.....	27.6	22.5	58.6	35.5	32.9	63.0

TABLE VI.

Body Weights (Less Gastrointestinal Weight) and Lengths Expressed as a Percentage of the Control Weights and Lengths at the End of the Experiments.

		Males.		Females.	
		Controls.	High protein.	Controls.	High protein.
		per cent	*per cent*	*per cent*	*per cent*
Series 1.	Weight.	100	75	100	74
	Length.	100	96	100	96
Series 2.	Weight.	100	82	100	75
	Length.	100	97	100	100

in each rat. With the single exception mentioned above the concentrations varied very little within each group. The averages are given in Table V.

The results in the control and cystine experiments are close to the averages we have compiled from observations on year old rats on a standard diet almost identical with the control diet used in these experiments. The higher level attained in the high protein experiments is a result of the large nitrogen consumption and parallels a similar effect observed in man on high protein diets

(5). It is only a transient phenomenon, for the blood urea quickly falls to the usual level when food is withheld. In the males in Series 1 it will be noted that the concentration is much lower than in the other high protein groups. In this case urine had been collected just before they were killed and they had consequently taken no food for 12 hours.

On postmortem examination the principal cause of the relatively low body weight of the high protein rats was found to be an absence of fat. This had indeed been evident during life, but it was strikingly demonstrated when the abdominal cavity was

TABLE VII.

Organ Weights per 100 Mm. of Body Length.

		Males.			Females.		
		Controls.	Cystine.	High protein.	Controls.	Cystine.	High protein.
Series 1.	Length, *mm.*	240	246	229	214	214	206
	Weight, *gm.*	161	164	126	126	118	94
	Liver, "	5.16	5.06	3.82	4.37	4.08	3.26
	Heart, *mg.*	446	428	455	351	376	364
	Kidneys, "	896	935	1060	697	755	850
Series 2.	Length, *mm.*	244	249	237	213	218	212
	Weight, *gm.*	170	169	143	118	99	92
	Liver, "	5.64	5.69	4.69	4.34	4.00	3.45
	Heart, *mg.*	449	443	449	370	345	372
	Kidneys, "	973	1000	1154	741	725	864

opened, for instead of the heavy layer of postperitoneal fat seen in the control and cystine rats only a small shrunken pad was left around the kidneys and elsewhere the bare muscles could be seen through the peritoneum. Whether all of the difference in the weights of the high protein and control rats can be accounted for by a failure to store fat is uncertain, but a comparison of the weights and lengths expressed as a percentage of the control weights and lengths suggests that this one difference is almost enough to account for the discrepancy. (Table VI.)

The complete postmortem records show that no gross abnormalities were found in the control rats with the exception of two instances of lipomata in the mesentery.

Among the cystine rats there was one with a calculus in the right kidney and another with the chronic bronchopneumonia

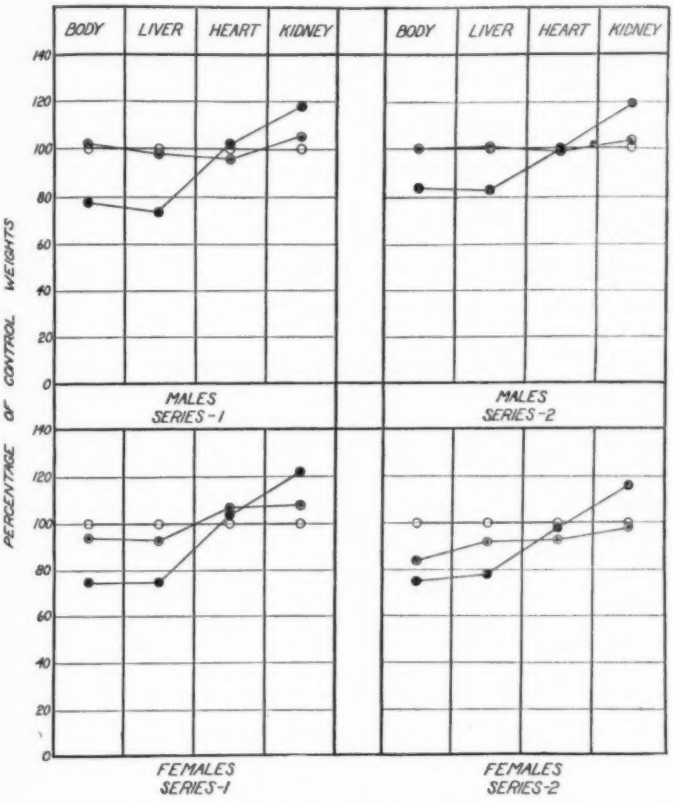

FIG. 6. Weights of the body, liver, heart, and kidney of rats on the cystine and high protein diets, expressed in relation to the same weights from rats on the control diet. Weights in the control rats per 100 mm. of body length = 100. Control diet = ○, cystine diet = ⊙, high protein diet = ●.

described by Hektoen (6), a condition which Donaldson (2) has found to be very commonly present in all old rats.

In the high protein rats which were kept on the diet for 330 days there was 1 with middle ear disease and 2 with unilateral hydronephrosis. In the 360 day groups 1 male had renal calculi with hydronephrosis. Of the 6 females in the series 5 had varying degrees of pneumonia. The "wheezy" breathing characteristic of the more advanced stages of this condition had been noted shortly before they were killed, and this lung condition is probably responsible for the loss of body weight observed during the last 20 days of life in this experiment.

On microscopical examination no difference could be detected between the kidneys of the control, cystine, and high protein groups. Careful measurements might have shown an increase in the size of the proximal convoluted cells and of the glomeruli in the high protein rats but it was not apparent at a glance.

The weight of the body minus the gastrointestinal tract, and the weights of the liver, heart, and kidneys were expressed as gm. or mg. per 100 mm. of body length (from the snout to the anus). The results are given in Table VII.

In order to make the weights observed in different experiments comparable with one another, the control weights have been taken as representing 100 per cent and the weights in the corresponding cystine and high protein expressed in proportion. These figures are charted in Fig. 6.

The only significant deviations as determined from a comparison of the actual differences from the control with the "probable differences" are in the high protein experiments. The weights of the body as a whole and of the liver are much lower than can be accounted for by chance while the kidney weights are considerably higher than the outermost range of accidental variation.

DISCUSSION.

All the precautions observed in these experiments have been specified with what may have seemed an unnecessary attention to detail. This is the result of an attentive reading of the literature on the effects of high protein diets. For it may be said that there would probably be much less difficulty in understanding the diversity of the results which have been obtained if all the significant features of the various experimental procedures had been fully described. As it is the lack of detail has been such

that those who have failed to find pathological changes in the kidney have been able to suggest that the lesions which were found may very well have been caused by other factors besides the high concentration of protein in the food. Indeed it seems very likely that certain of the high protein diets which have been used have also been defective diets. We do not think that this is the only reason for the difference in results, but as several investigators have not yet published their work in full, we shall not discuss these possibilities but shall content ourselves with saying that in so far as damage to the kidney from high protein diets is concerned, we are in agreement with the statement in the preliminary paper of Osborne, Mendel, Park, and Darrow (7) and with the reports of Drummond, Crowden, and Hill (8), of Reader and Drummond (9), of Miller (10), and of Jackson and Riggs (11) to the effect that no deleterious structural effects were produced. We can also fully confirm the conclusion of Jackson and Riggs that a study of the urine and blood gives no indication of any renal abnormality. On the other hand renal lesions which appear to be degenerative in nature have been found in rabbits kept on a diet containing a moderate excess of protein by Newburgh (12) and by Polvogt, McCollum, and Simmonds (13) and by Evans and Risley (14) in rats. Newburgh, Marsh, Clarkson, and Curtis (15) have also observed evidences of the existence of pathological changes in the kidneys of rats to whose food even less than 1 per cent of cystine had been added. These results are important because they are positive. Our negative findings are not directly opposed, since we did not attempt to duplicate their conditions, nor are our results so general in nature that they can be regarded as final. But they do quite decisively prove that rats can live for a third of their life span on a diet very high in casein or with added cystine and yet escape any damage to their kidneys. This fact leads us to reject the hypothesis that a large consumption of protein is in and by itself necessarily harmful to the kidneys.

The only constant effects of these diets, if we except the interesting but obscure influence of the high protein diet on the rate of protein excretion, was a slight hypertrophy of the kidney, a decrease in the weight of the liver, and a failure to store fat when the bulk of the food consisted of casein.

CONCLUSIONS.

1. Rats which lived for one-third of their life time on a diet which contained 70 per cent of protein grew nearly to the same length as the controls but had a lower body weight as a consequence of an almost complete absence of fat. There was an increase in the weight of the kidney and a decrease in the weight of the liver.

2. No pathological changes were found in the urine or in the kidneys of the rats which had lived on 70 per cent protein, or on a diet which contained 1 per cent of cystine.

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XUM

THE EFFECT ON THE KIDNEY OF THE LONG CONTINUED ADMINISTRATION OF DIETS CONTAINING AN EXCESS OF CERTAIN FOOD ELEMENTS.

II. EXCESS OF ACID AND OF ALKALI.*

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From time to time it has been suggested that the long continued consumption of food which contains an excess of acid may ultimately lead to the development of pathological changes in the kidney. Sansum, Blatherwick, and Smith (1) have adopted this hypothesis and have advocated the use of "basic diets" in the treatment of Bright's disease and arteriosclerosis, and Nuzum, Osborne, and Sansum (2) observed a rise of blood pressure and the appearance of atherosclerosis and of renal lesions in rabbits kept for a long time on acid diets. There is thus reason for an investigation into the effect on the kidney of an excess of acid and of basic radicals, quite apart from the interest of a study of their influence on nutrition in general.

We have used the methods which are given in detail in the preceding paper. The animals there described as controls serve the same purpose for the experiments reported here.

The acid diet was made by thoroughly mixing 2 gm. of $CaCl_2$ with every 98 gm. of the control diet whose exact composition has already been explained. The grains of the finely powdered $CaCl_2$ evidently became covered with a film of oil in the process of mixing, for there was no weight change when the food was exposed to air. Haldane, Hill, and Luck (3) showed that the oral administration of $CaCl_2$ is equivalent in effect to giving HCl because most of the Ca remains in the intestine while the Cl is absorbed. In a quantitative study of this phenomenon Gamble, Ross, and Tisdall (4) demonstrated that about 40 per cent of the chlorine in

* This work was aided by the Wellington Gregg Fund for the investigation of Bright's disease.

$CaCl_2$ acted within the body as HCl. If we accept this as true for the rat, 100 gm. of the $CaCl_2$ diet would contain the equivalent of 144 cc. of 0.1 N HCl. A man who took enough of this diet to provide 2500 calories would consume the equivalent of 768 cc. of 0.1 N HCl daily.

The alkaline diet was made by mixing 4 gm. of $NaHCO_3$ with each 96 gm. of the control diet. For man this food would involve the consumption every day of about 2560 cc. of 0.1 N NaOH.

TABLE I.

Average Percentages of Total N_2 Excreted as Ammonia.

	Total urine N_2. Mg. per rat per 24 hrs.	Ammonia N_2. Mg. per rat per 24 hrs.	Ammonia N_2. Per cent of total N_2.
Controls (cystine)	138	6.2	7.5
Acid diet	188	12.9	11.4
Alkaline diet	120	2.7	3.8

TABLE II.

Averages of 12 Hour Rates of Excretion in Urine from Series 1 and 2 Expressed as Rates per Gm. of Renal Tissue.

	Male.			Female.		
	Control.	Acid.	Alkaline.	Control.	Acid.	Alkaline.
Volume, *cc.*	1.2	1.2	1.6	1.3	1.2	1.9
Protein, *mg.*	2.2	1.1		0.7	0.4	0 8
White and epithelial cells	24,000	20,000	32,000	30,000	20,000	46,000

In other experiments with these diets we had found that the average reaction of the urine was pH 6.4 with the control diet, 5.2 with the acid diet, and 6.9 with the alkaline diet. It may be that similar differences in reaction were not maintained in the present experiments throughout their whole duration, for towards their close ammonium-magnesium-phosphate crystals were sometimes seen in the urinary sediment of the rats on the acid diet. But whatever adjustment may have occurred the measurements of the percentages of total nitrogen excreted as ammonia, which were obtained shortly before the animals were killed, indicate that even to the end the $CaCl_2$ diet was exerting an acid and the

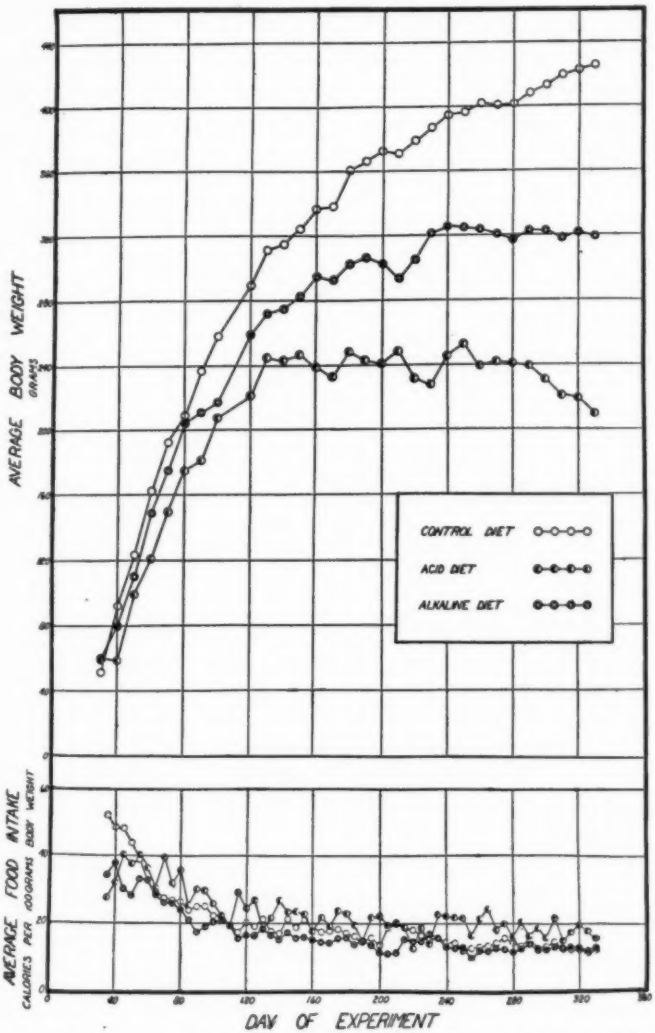

FIG. 1. Average body weight and food consumption of male rats of the first series of experiments (330 days). Control diet = ○, acid diet = ●, alkaline diet = ⊙.

$NaHCO_3$ diet an alkaline effect on the form of nitrogen excretion (Table I).

Two series of experiments were completed, the first running from 30 days to 330 days of age and the second from 30 days until the

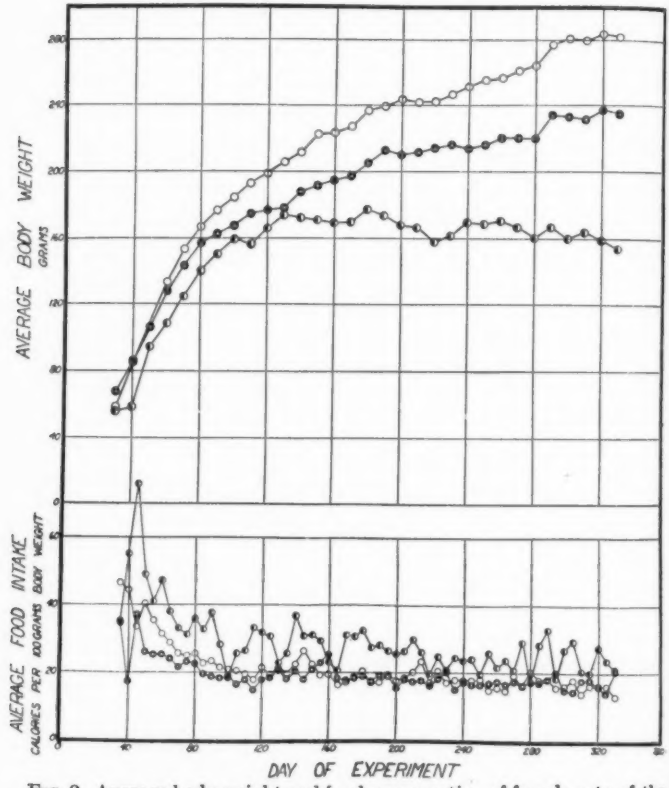

FIG. 2. Average body weight and food consumption of female rats of the first series of experiments (330 days). Control diet = ○, acid diet = ●, alkaline diet = ⊙.

animals were 360 days old. The body weight and food consumption curves for male and female rats for both series are given in Figs. 1 to 4.

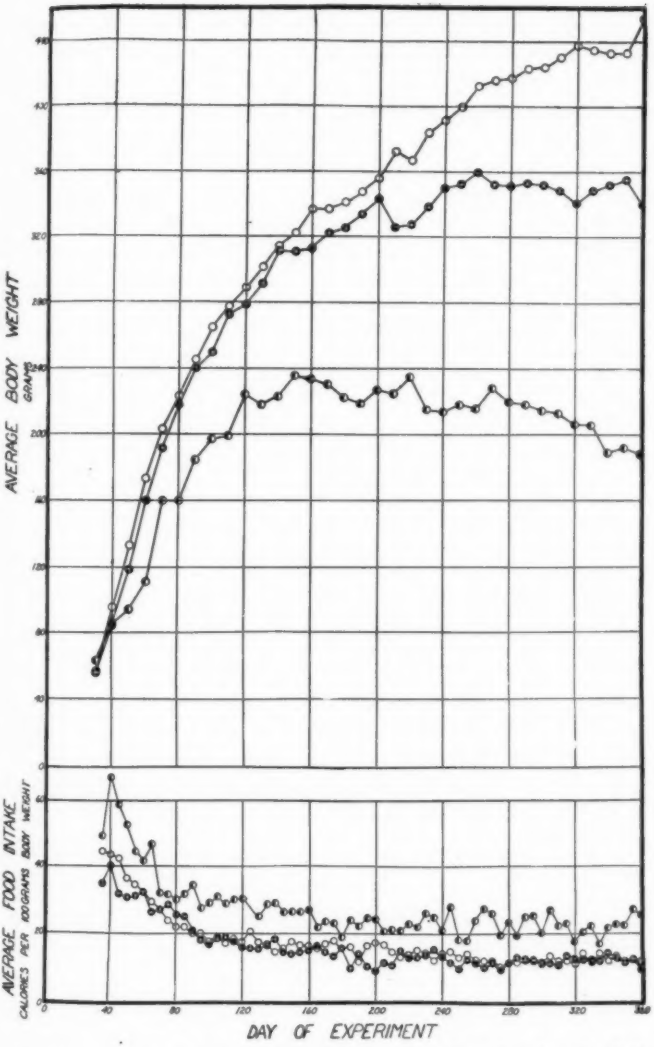

FIG. 3. Average body weight and food consumption of male rats of the second series of experiments (360 days). Control diet = ○, acid diet = ◐, alkaline diet = ●.

The acid diet rats at first grew almost but not quite as quickly as the controls but from about the 120th day of age they ceased to gain and thereafter tended slowly to lose in weight. The food

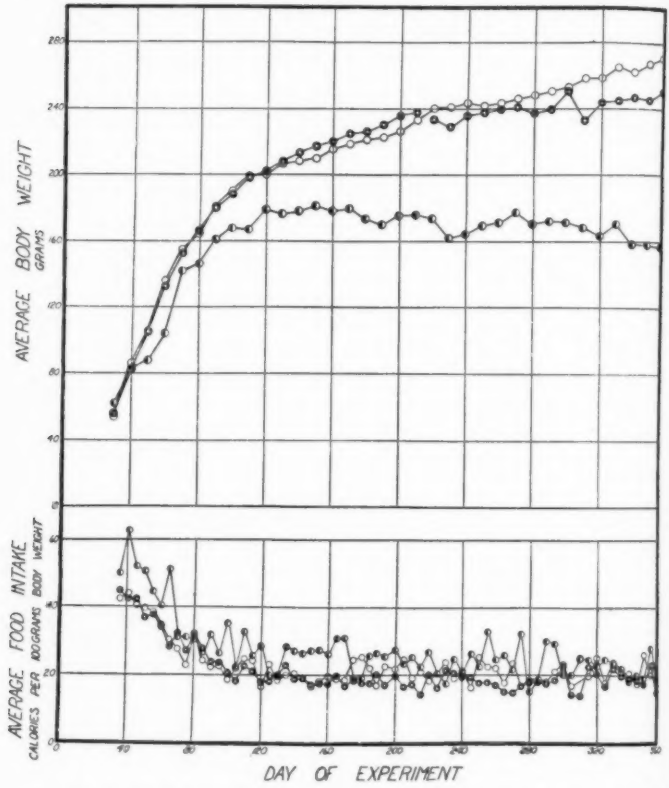

FIG. 4. Average body weight and food consumption of female rats of the second series of experiments (360 days). Control diet = ○, acid diet = ◐, alkaline diet = ●.

graph reveals the paradoxical circumstance that not only is this not to be explained by an insufficient food supply but that on the contrary the inhibition of growth and failure of maintenance occurs in spite of a food intake which considerably exceeds that of the controls.

The alkaline diet rats continued to gain in weight throughout the whole period of observation but though they took as much to eat as the controls their rate of increase in weight was less rapid.

The average urine volumes, amounts of protein, and number of white blood and epithelial cells expressed as 12 hour rates per gm. of renal tissue are given in Table II.

Apart from the slight increase in urine volume and in the number of white blood and epithelial cells in the urine of the rats on the alkaline diet, the only striking fact in this table is the decrease in the rate of protein excretion in the urine of the acid diet rats. In all the four experiments the amount of protein eliminated by these animals is only about half of the quantity excreted by the controls. The decrease cannot be accounted for simply as a result of the absence of blood in the urine of this group.

TABLE III.

Average Blood Urea Concentrations. Mg. per 100 Cc.

	Male.			Female.		
	Control.	Acid.	Alkaline.	Control.	Acid.	Alkaline.
Series 1.....	30.2	30.5	36.1	33.5	36.3	40.9
" 2.....	27.6	43.3	35.7	35.5	44.0	36.1

As in the preceding experiments an occasional cast was now and then discovered but no more frequently in one group than in another.

A difference in the amount of blood in the urine was the most pronounced and constant effect associated with these diets. In the control group, as has already been shown, there were 2 urines of the 78 examined in which the presence of blood was obvious, and on microscopical examination some red cells were usually to be found. But in the alkaline diet urines 24 of the 72 specimens contained large amounts of blood. With one exception these instances of gross hematuria occurred in male rats. But even in the urine from the females and in the apparently normal urines from the males hundreds of thousands of red cells were found. In sharp contrast to the hemorrhagic urines of the alkaline diet rats were the uniformly clear amber-colored collections obtained from the animals on the acid diet. The distinction was only deepened

by the microscopical examination for in not one of the urines obtained from these 24 rats was there a single red blood cell to be seen.

The blood urea concentrations measured at the conclusion of the experiments were in all cases within the range of normal variation. The averages are given in Table III.

No gross abnormalities were found at postmortem examination in any of the rats on the acid diet. There was perhaps somewhat less fat than in the controls, but the difference in the weight was due in the main to the generally reduced bulk of the animals.

TABLE IV.

Organ Weights per 100 Mm. of Body Length.

		Males.			Females.		
		Controls.	Acid.	Alkaline.	Controls.	Acid.	Alkaline.
Series 1.	Length, *mm.*	240	218	231	214	199	212
	Weight, *gm.*	161	88	126	126	70	100
	Liver, "	5.16	4.12	4.04	4.37	3.46	3.67
	Heart, *mg.*	446	344	415	351	298	345
	Kidneys, "	898	941	885	697	707	732
Series 2.	Length, *mm.*	244	215	233	213	203	212
	Weight, *gm.*	170	76	136	118	69	108
	Liver, "	5.64	3.92	4.31	4.34	3.46	3.49
	Heart, *mg.*	449	338	400	370	299	347
	Kidneys, "	973	955	964	741	747	748

In the rats on the alkaline diet there were 7 of the 24 in which hydronephrosis of one or both kidneys was present, although in no instance was the condition so far advanced as to have produced an appreciable renal atrophy. The dilated pelves were full of bloody urine. Apart from the mechanical effects of the hydronephrosis there were no apparent microscopical differences between the kidneys of the control, acid, and alkaline rats. The organ weights per 100 mm. of body length are given in Table IV and these figures expressed as a percentage of the control weights have been charted in Fig 5.

In both the acid and alkaline diet rats there is a significant reduction in the weight of the liver, but no noteworthy change in

the kidney weights. The pronounced decrease in the weight of the heart in all the acid experiments is curious and quite unex-

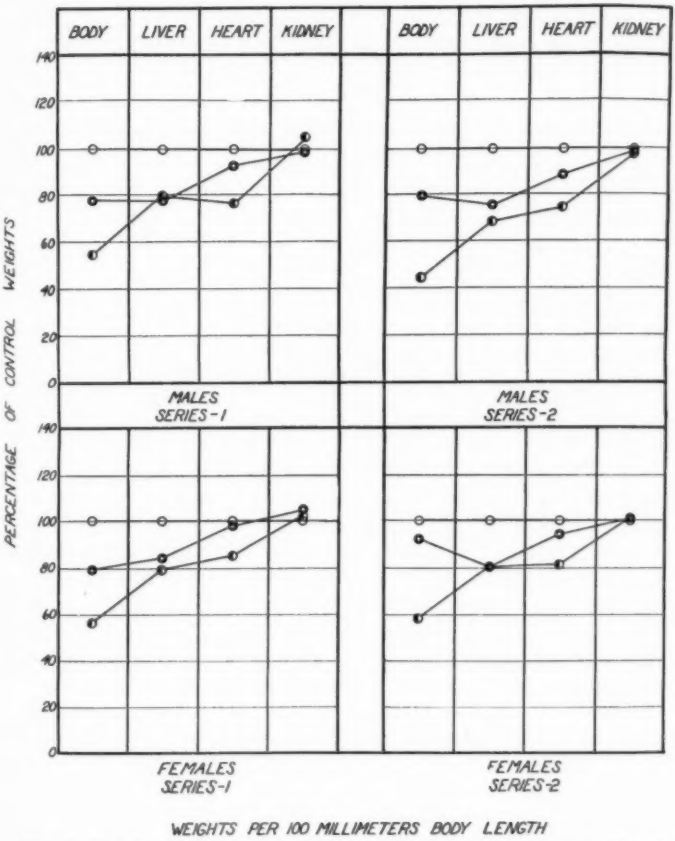

FIG. 5. Weight of the body, liver, heart, and kidney of rats on acid and on alkaline diets expressed in relation to the same weights from rats on the control diet. Weights in the control rats per 100 mm. of body length = 100. Control diet = ○, acid diet = ◐, alkaline diet = ●.

plained. It appears to be a specific effect of the acid diet for on all the other diets, high protein and cystine as well as alkaline, the heart weight remains constant.

Whatever may be the immediate cause of the disproportionate diminution in the size of the heart in this group, the fact as such stands in opposition to the hypothesis advanced by Nuzum and his collaborators that his acid diets produced an increase in blood pressure by virtue of their acidity.

CONCLUSIONS.

1. No pathological changes were found in the urine or in the kidneys of albino rats after they had lived for about one-third of their average life time on a diet which was made acid by the addition of calcium chloride.

2. Hydronephrosis was found in 7 of the 24 rats fed for a long time on a diet which contained 4 per cent of sodium bicarbonate.

3. Red blood cells in small numbers were frequently found in the urine of the rats on the control diet, but no red blood cells were ever seen in the urine of the rats on the calcium chloride diet. On the other hand the urine of the rats on the sodium bicarbonate diet was often red with blood and large numbers of red blood cells were always found on microscopical examinations.

4. The rate of protein excretion in the urine of the rats on the acid diet was reduced to about half of the quantity measured in the urine of the rats on the control diet.

5. In the rats on the acid diet the body weight and the weight of the heart per 100 mm. of body length was much less than in the controls. The weight of the liver was decreased in the rats from both the acid and the alkaline diet but the kidney weights were not appreciably different from the values obtained in the controls.

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2. Nuzum, F. R., Osborne, M., and Sansum, W. D., *Arch. Int Med.*, 1925, xxxv, 492.
3. Haldane, J. B. S., Hill, R., and Luck, J. M., *J. Physiol.*, 1923, lvii, 301.
4. Gamble, J. L., Ross, G. S., and Tisdall, F. F., *Am. J. Dis. Child.*, 1922, xxv, 455.

XUM

THE ACTION OF HYDRAZINE HYDRATE ON URIDINE.

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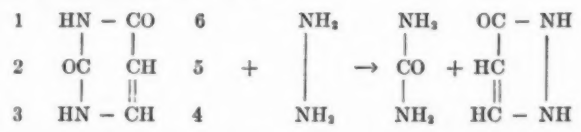
(Received for publication, October 29, 1926.)

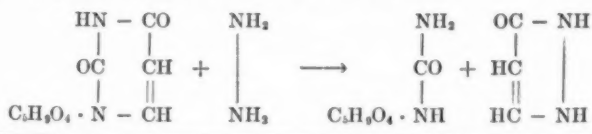
In previous communications[1] from this laboratory, evidence has been submitted supporting the view that in the pyrimidine nucleosides the sugar molecule is linked to nitrogen atom (3). We are now able to report in favor of this structure additional, more conclusive evidence based on an investigation of the action of hydrazine on uridine.

It has recently been shown[2] that hydrazine reacts with uracil to form urea and pyrazolone,



the two products of the reaction being isolated as xanthyl derivatives. Analogously, thymine yields urea and 4-methylpyrazolone.

An extension of this reaction to uridine should theoretically lead to definite evidence in regard to the position of the sugar linkage. If the sugar is in position (3), we may expect to obtain as primary products pyrazolone and urea riboside:



[1] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608. Levene, P. A., *J. Biol. Chem.*, 1925, lxiii, 653. Levene, P. A., Bass, L. W., and Simms, H. S., *J. Biol. Chem.*, 1926, lxx, 229.

[2] Fosse, R., Hieulle, A., and Bass, L. W., *Compt. rend. Acad.*, 1924, clxxviii, 811.

On the other hand, if the sugar is linked to carbon atom (4), we may expect to obtain urea and the ribose derivative of pyrazolone. The products indicated in one or the other of these reactions should theoretically be formed, provided that the linkage between the sugar and the pyrimidine is not broken by the action of hydrazine.

If ribose were linked to carbon atom (4) of the pyrimidine, it is reasonable to assume *a priori* that the union would resist the action of hydrazine, since in this case the sugar would be united to the pyrimidine by a carbon to carbon bond.

It is difficult, however, to predict on theoretical grounds whether the sugar would be split off by the action of hydrazine if it were united to the pyrimidine in position (3). It was therefore necessary to test this possibility experimentally.

Fischer and Helferich[3] and Levene and Sobotka[4] were unsuccessful in their attempts to prepare pyrimidine glucosides with the sugar attached to a nitrogen atom. Since no suitable pyrimidine derivative was available, experiments were performed on urea glucoside, inasmuch as uracil may be regarded as a substituted urea. These experiments proved, first, that this glucoside is not hydrolyzed by hydrazine, since it is not possible to isolate urea from the reaction product by means of xanthydrol, whereas urea is readily isolated from a mixture of urea, glucose, and hydrazine. Second, it was shown that urea glucoside reacts with hydrazine to form a product which does not yield an insoluble xanthyl derivative. This conclusion was drawn from the following experimental results. When urea glucoside is treated with xanthydrol in acetic acid solution, it forms a characteristic monoxanthyl derivative (unsubstituted urea yields a dixanthyl derivative). However, if urea glucoside is first warmed with hydrazine, neither xanthyl urea glucoside nor dixanthylurea can be isolated from the reaction product.

On the basis of these considerations the following conclusions were formulated:

1. The isolation of a ribose-free pyrazolone derivative from the product of the reaction between uridine and hydrazine may be regarded as an indication that ribose is linked to the pyrimidine in position (3).

[3] Fischer, E., and Helferich, B., *Ber. chem. Ges.*, 1914, xlvii, 210. Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 1377.

[4] Levene, P. A., and Sobotka, H., *J. Biol. Chem.*, 1925, lxv, 469.

2. The isolation of free urea may be regarded as evidence in favor of the linkage in position (4).

From this standpoint, the experiments on uridine afforded convincing evidence in favor of the structure which assumes a union of the sugar with nitrogen atom (3) of the pyrimidine. From the reaction product, ribose-free pyrazolone was isolated in the form of its dixanthyl derivative. Furthermore, it was shown that free urea was not formed in appreciable quantity (if at all). Further efforts are being directed toward the isolation of the hydrazine derivative of urea riboside.

SUMMARY.

1. It has been shown by Fosse, Hieulle, and Bass that uracil reacts with hydrazine to form urea and pyrazolone.

2. Urea can be isolated by means of xanthydrol from a mixture containing urea, glucose, and hydrazine.

3. Urea glucoside forms a difficultly soluble monoxanthyl derivative.

4. From the reaction product of hydrazine and urea glucoside neither free urea nor urea glucoside can be isolated by means of xanthydrol.

5. From the reaction product of hydrazine and uridine a ribose-free derivative of pyrazolone, but no perceptible quantities of urea, can be isolated.

6. It is therefore concluded that no perceptible quantity of ribose is liberated from uridine by the action of hydrazine, and hence, that the isolation of ribose-free pyrazolone is an indication that in uridine the union of ribose to uracil is in position (3) of the pyrimidine. This conclusion is further supported by the failure to isolate an appreciable quantity of urea.

EXPERIMENTAL.

Action of Hydrazine on Uridine.

0.4 gm. of uridine was heated in a stoppered test-tube with 1.00 cc. of hydrazine hydrate[5] at 65°C. for 1 hour. The reaction mixture, which consisted of a turbid, light brown solution, was

[5] Kahlbaum hydrazine hydrate for analysis.

washed into a flask with 13.0 cc. of water and 14.0 cc. of glacial acetic acid. To this solution was added a solution of 1.4 gm. (> 4 mols)[6] of xanthydrol in 14.0 cc. of glacial acetic acid. After standing overnight, the precipitate was filtered off by suction and was then washed with water, alcohol, and again with water. The dried product, after recrystallization from acetone, melted at 210–213°C. The recrystallized material, after drying at 100°C. under reduced pressure over sulfuric acid, gave an analysis corresponding to dixanthylpyrazolone.

No. 520. 0.1018 gm. substance: 0.2910 gm. CO_2 and 0.0420 gm. H_2O.
0.1455 " " : (Dumas) 7.80 cc. N_2 at 21°C. and 769 mm.
$C_{29}H_{20}O_2N_2$. Calculated. C 78.38, H 4.54, N 6.30.
Found. " 77.95, " 4.64, " 6.32.

In a series of experiments, each with 0.40 gm. of uridine, the precipitate obtained by means of xanthydrol weighed 0.50 to 0.70 gm. Assuming that the precipitate consisted entirely of dixanthylpyrazolone, these values correspond to 0.09 to 0.13 gm. of pyrazolone,[7] or 0.27 to 0.38 gm. of uridine.

Although urea is precipitated quantitatively[8] by xanthydrol under these conditions, none of these precipitates appeared to contain dixanthylurea, the strongest proof of this conclusion being their complete solubility in comparatively small volumes of acetone. In this connection it should be noted that in the work on uracil[2] the isolation of dixanthylurea was accomplished by extracting the dixanthylpyrazolone from the mixture by means of acetone.

To substantiate our conclusion that these two xanthyl derivatives could be separated by fractional solution in acetone, it was necessary to determine approximately their solubilities in this solvent. The solubility of dixanthylpyrazolone is roughly 0.2 gm. per 100 cc. of acetone at the boiling point, while the solubility of dixanthylurea is less than 0.008 gm. per 100 cc.

A second series of experiments with uridine was then performed

[6] Four mols of xanthydrol were employed to insure complete precipitation of the possible products of the reaction; *i.e.*, urea and pyrazolone.

[7] It is not known whether pyrazolone is precipitated quantitatively by xanthydrol.

[8] Fosse, R., *Ann. Inst. Pasteur*, 1916, xxx, 525.

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according to the following procedure. 0.20 gm. of the nucleoside was heated with 1.00 cc. of hydrazine at 65°C. for 1 hour. The precipitation with 4 mols of xanthydrol was carried out as in the preceding experiments. The precipitates weighed 0.25 to 0.28 gm. (≡ 0.048 to 0.053 gm. of pyrazolone ≡ 0.14 to 0.15 gm. of uridine), and were completely soluble in 100 to 125 cc. of acetone. Hence, if dixanthylurea were present, the quantity was less than 0.01 gm. (≡ 0.001 gm. of urea ≡ 0.006 gm. of uridine).

Isolation of Urea from Reaction Mixtures Containing Hydrazine and a Sugar.

It has already been shown in the investigation of the action of hydrazine on uracil[2] that urea can be isolated from a reaction mixture containing pyrazolone and excess hydrazine. In order to comply with the conditions of our experiments, it was necessary to prove that urea can also be isolated from a reaction mixture containing hydrazine and a sugar.

0.1 gm. of urea was heated with 0.5 cc. of hydrazine at 65°C. for 1 hour. From the reaction mixture the urea was precipitated as dixanthylurea. After recrystallization from pyridine it melted at 263°C. For analysis it was dried at 100°C. under reduced pressure over sulfuric acid.

No. 515. 0.1000 gm. substance: 0.2830 gm. CO_2 and 0.0426 gm. H_2O.
0.1610 " " : (Dumas) 9.50 cc. N_2 at 28°C. and 759 mm.
$C_{27}H_{20}O_2N_2$. Calculated. C 77.14, H 4.81, N 6.66.
Found. " 77.17, " 4.77, " 6.70.

A mixture of 0.1 gm. of urea and 0.3 gm. of glucose was heated with 0.5 cc. of hydrazine at 65°C. for 1 hour. The dixanthylurea was precipitated and purified as in the preceding experiment. It melted at 263°C. and gave the following analysis.

No. 516. 0.0979 gm. substance: 0.2786 gm. CO_2 and 0.0420 gm. H_2O.
0.1562 " " : (Dumas) 9.00 cc. N_2 at 25°C. and 761 mm.
$C_{27}H_{20}O_2N_2$. Calculated. C 77.14, H 4.81, N 6.66.
Found. " 77.60, " 4.80, " 6.60.

A similar result was obtained when ribose was substituted for glucose.

Xanthyl Urea Glucoside.

Urea glucoside was prepared according to the methods of Schoorl[9] and Helferich and Kosche.[10]

The xanthyl derivative was prepared by adding to a solution of 2.0 gm. of the ureide in 50.0 cc. of 50 per cent aqueous acetic acid a solution of 2.0 gm. (> 1 mol) of xanthydrol in 25.0 cc. of glacial acetic acid. The solution showed a yellow color for an instant, then became very viscous, and in a few minutes had become a heavy gel. This gel was very difficult to filter and attempts to purify the solid by recrystallization were unsuccessful. For purification it was washed repeatedly with alcohol on a suction filter. It was dried at 100°C. under reduced pressure over sulfuric acid.

No. 365. 0.0975 gm. substance: 0.2124 gm. CO_2 and 0.0516 gm. H_2O.
0.1159 " " : (Dumas) 7.00 cc. N_2 at 23°C. and 748 mm.
$C_{20}H_{22}O_7N_2$. Calculated. C 59.70, H 5.52, N 6.96.
Found. " 59.40, " 5.92, " 6.85.

It melted with decomposition at 216–219°C.

The substance was found to have a low levorotation in saturated solution in acetic acid but no accurate reading could be obtained.

Action of Hydrazine on Urea Glucoside.

0.4 gm. of urea glucoside was heated with 0.50 cc. of hydrazine and 1.00 cc. of water at 65°C. for 1 hour. On cooling, the clear solution solidified. A test with xanthydrol showed that neither urea glucoside nor free urea was present.

[9] Schoorl, N., *Rec. trav. chim. Pays-Bas*, 1903, xxii, 31.
[10] Helferich, B., and Kosche, W., *Ber. chem. Ges.*, 1926, lix, 69.

XUM

THE PYRIDINE TEST AS A QUANTITATIVE METHOD FOR THE ESTIMATION OF MINUTE AMOUNTS OF CHLOROFORM.*

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(Received for publication, October 20, 1926.)

I.

In spite of many investigations concerning the distribution and fate of anesthetic and lethal doses of chloroform in animal fluids, tissues, and organs,[1] there have been relatively few accurate quantitative determinations of the amounts of chloroform in various parts of the animal body following chloroform poisoning. The most reliable data are evidently those reported by Buckmaster and Gardner (1906, 1907) for blood, and by Nicloux and Yovanovitch (1924, 1925) for nervous tissues and blood. The obvious reason for the absence of such determinations is the lack of any simple quantitative method whereby minute amounts of chloroform can easily be measured. This lack also explains why satisfactory studies on the permeability of animal and plant membranes to chloroform have not been made. Although there are several qualitative tests for chloroform, such as the phenylisocyanide, resorcinol, naphthol, cyanide, and reduction tests (see Autenrieth and Warren, 1921, pp. 36–38), none of these is delicate enough to determine minute amounts, and some of them are too elaborate to be of much practical value. The important quantitative tests are those which involve the decomposition of chloroform and the subsequent determination of chlorine, either by gravi-

* A part of the expense of this investigation was defrayed by a grant from the American Association for the Advancement of Science.

[1] Some of the more important studies include those by Grehant and Quinquand, 1883; Luedeking, 1886; Pohl, 1890–91; Angiolani, 1891; Nicloux, 1906, 1907; Nicloux and Yovanovitch, 1924; Buckmaster, 1917–18; Buckmaster and Gardner, 1906, 1907; all of which are listed in the Bibliography of this paper.

metric or volumetric methods, as in those of Ludwig-Fisher,[2] Nicloux (1906, 1924), Gibson and Laidlaw (1922), Buckmaster and Gardner (1906, 1907), and others; all of these are laborious without being very delicate. Several years ago, however, Fujiwara (1914, 1917) described the pyridine reaction with chloroform and related substances in the presence of strong alkali, stating that 1 part of chloroform in a million parts of water could easily be detected. Later, Ross (1923–24) reported the same test, indicating the independent discovery that substances containing the R-C-halogen$_3$ group (chloroform, bromoform, chloretone, chloral, iodoform, etc.) give a pink or red color to pyridine when the mixture is heated in the presence of sodium hydroxide. Fujiwara applied the test to extracts and distillates of animal fluids and tissues, attempting to estimate the amount of chloroform present by the depth of the color produced. These experiments suggested to the writer the possibility of developing the pyridine reaction quantitatively, so that accurate determinations of the amounts of chloroform and related substances present in solutions might be made. The value of such a simple and delicate test for studies on the permeability of membranes to chloroform, on the distribution of chloroform in the tissues of animals anesthetized or killed by chloroform, as well as for the determinations of chloroform poisoning in medicolegal practice, is obvious.

II.

For quantitative estimations of chloroform, the procedure has been standardized and found satisfactory for measuring the amounts between 0.1 and 0.0001 per cent, or 0.01256 and 0.00001256 molar concentrations, when the specific gravity is 1.5. Future experiments will deal with the test as applied to the other substances containing the R-C-halogen$_3$ group. The procedure in the case of chloroform is as follows. Into a narrow test-tube of about 10 cc. capacity are measured 2 cc. of 20 per cent NaOH from a long burette graduated to 0.1 cc. To this are added 1 cc. of chemically pure pyridine (colorless) from a similar burette, and 1 cc. of the solution to be tested, measured by a 1 cc. pipette. The test-tube is loosely corked to prevent undue evaporation of

[2] See Autenrieth and Warren, 1921, p. 38.

the pyridine, and, with constant shaking, is immersed well above the liquid level for 1 minute in water at 100°C. The tube is transferred to cold water until the temperature of the mixture has fallen to about 20°C. During the cooling, the pyridine and test solution, colored pink or red if chloroform is present, rise above the alkali, and are removed by a 1 cc. pipette to a color comparison tube. The solution may then be compared with color standards previously made up to match the colors obtained from known concentrations of chloroform. For quantitative work it is necessary that the amounts of pyridine and test solution be measured accurately, since the color produced is a function of the volume over which it is distributed. Moreover, since pyridine is volatile (b. p. about 115°C.), the heating should be done in long loosely corked tubes, so as to keep the volume of pyridine as constant as possible. The color comparison tubes must be of the same diameter and of the same kind of glass as those used for the color standards. When all directions are followed accurately, the color from any one concentration of chloroform may be duplicated regularly.

Since the colored pyridine compound is unstable and gradually loses its color, it cannot be used as the standard. It has been found that basic fuchsin (certified by the Commission on Standardization of Biological Stains) may be used to match the colors obtained from all the higher concentrations of chloroform with considerable accuracy. For the more dilute solutions it does not match exactly, but comes nearer than any other dye so far found.[3] The basic fuchsin standards were made up with 50 per cent alcohol containing about 0.01 per cent HCl, and enough dye to match the desired color.

A 0.1 per cent stock solution of chloroform was made by dissolving 1 cc. of Squibb's chemically pure chloroform, sp. gr. 1.500, at 15°C., measured by a certified pipette graduated in 0.1 and 0.01 cc., in distilled water and making up to 1000 cc. in a volumetric flask at 15°C. The other solutions were made by diluting the stock solution with varying amounts of distilled water, using large volumes to insure greater accuracy. All of these solutions were kept in ground glass-stoppered bottles whose volumes were

[3] The firm of Hynson, Westcott and Dunning of Baltimore kindly made up an experimental set of color standards, for which the author expresses his thanks.

only slightly in excess of the solution they contained. In making the stock chloroform solutions to be used as standards in matching the fuchsin color solutions, it is highly important that all measurements of volume be as accurate as possible, and that the temperature of all fluids be 15°C. Only certified pipettes, volumetric flasks, and thermometers should be used. These precautions are necessary because of the volatility of chloroform and the delicacy of the test. It was found that a nearly saturated solution of basic fuchsin in acidified alcohol matched the color obtained from the pyridine test on a 0.1 per cent chloroform solution. For the other color standards (0.05, 0.025, 0.01, 0.005, 0.003, 0.001, 0.00078, 0.0005, 0.0002, and 0.0001 per cent, corresponding to molar concentrations of 0.00628, 0.00314, 0.001256, 0.000628, 0.0003768, 0.0001256, 0.0000979, 0.0000628, 0.00002512 and 0.000012560) this stock fuchsin solution was diluted until it matched as closely as possible the colors obtained from known concentrations of chloroform. Each color standard was checked frequently and corrected whenever necessary.

The colored pyridine solution obtained from a test on a chloroform solution in water is only slightly turbid and may be compared directly with the color standards for concentrations of 0.1, 0.05, and 0.025 per cent. Below the latter concentration the turbidity interferes with the matching of colors, and two sets of tubes must be used in the comparator. One set consists of distilled water and the pyridine colored solution from the test; while the other consists of the pyridine mixture from a test on a solution containing no chloroform and the color standard tube. When extracts of tissues are used the double set of tubes is always necessary, an extract from tissues containing no chloroform being used in the control tube.

III.

Preliminary experiments were concerned with qualitative tests on extracts of animal organs and tissues in various solvents. The following were found satisfactory for making tests: physiological salt solution, Ringer's solution, acidified distilled water (about 0.01 per cent HCl), alcohol, ether, and tartaric acid-alcohol (70 per cent alcohol with 1 per cent tartaric acid). The only solvent, however, which will allow the detection of chloroform in the small-

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est amounts, is acidified distilled water (*cf.* Fujiwara, 1914, p. 3). This seems to be due to the facts that distilled water extracts from the tissues a larger percentage of the chloroform than the other solvents, and that the pyridine reaction is more delicate in water than in alcohol or ether. Slight acidity prevents any decomposition of the chloroform which might occur in an alkaline medium. Toluene may safely be used in small amounts as a preservative. On the other hand, it is perfectly clear that blood seriously interferes with the test. If considerable blood is present in the extract the pink color of the test will be hidden by an orange-brown turbidity. A moderate amount of blood in the test mixture produces an orange color which rapidly becomes orange-red, then brown, green-brown, and finally light green. In general, less than 5 minutes are required for the completion of this color series, the actual time depending upon the amount of blood present. If an extract contains only enough blood to give it a barely perceptible pink tint, the test may be made without interference, since control tests on such extracts yield a colorless pyridine solution in the absence of chloroform.

The following procedure was used in preparing the extracts. The organ is dissected out and submerged immediately in a mortar with a small amount of the solvent to be used. It is then cut up into small pieces by scissors, care being taken not to expose the tissue to the air. With a pestle the material is ground until it is thoroughly minced or shredded, and then the whole mass is placed in a rubber-stoppered flask with the desired amount of solvent. This amount is determined by the quantity of chloroform expected to be found. The extract should stand at room temperature for at least 1 hour, with occasional shaking, so that all the chloroform may be extracted. The pyridine tests are made within the next few minutes, since the longer the mixture stands after complete extraction the less accurate will be the estimation, due to the unavoidable loss of chloroform vapor. When enough blood is present to hinder the test, distillation is necessary. A small distilling flask (25 cc.) with a long neck, and a side arm surrounded by running cold water, has been used. The heating was done in a sand or an oil bath. The distillate is conducted into from 3 to 5 cc. of acidified distilled water in a small test-tube loosely corked. At the end of distillation, the distillate solution is tested. This

method has not yet been improved for reliable quantitative estimations of chloroform in the original extract.

The qualitative tests were made on extracts (1) from the tissues of animals killed by chloroform inhalation; (2) from tissues of animals killed by ether, illuminating gas, a blow on the head, electricity, and natural causes; as well as on (3) solutions containing chloroform which had diffused through frog skin. In the first and third groups positive tests were always found, indicating the presence of chloroform; while in the second group the tests were always negative. It was further found that slight decay of the tissues does not seriously interfere with the test. 200 tests were made on extracts from the tissues of frogs, mice, guinea pigs, rats, cats, dogs, and human beings, and in no case did the method fail to indicate chloroform when the latter was known to be present. It was concluded that the pyridine reaction is a reliable test for chloroform under the conditions stated.

IV.

For quantitative tests the concentrations of chloroform mentioned (p. 176) were carefully prepared by the writer, and the bottles were given non-successive arbitrary numbers by a disinterested person. Each solution was then tested three times by the pyridine reaction, and the estimated concentration of chloroform was recorded under the corresponding number of the bottle. Although this scheme was used many times there was less then 1 per cent discrepancy, and all of this was restricted to the last three most dilute solutions. It appears that the pyridine test is a reliable quantitative method for estimating minute amounts of chloroform in aqueous solutions.

As a further check on the method, extracts of the brains of rats killed by illuminating gas were prepared in the described manner, and to the different extracts the following amounts of chloroform were added: 0.1, 0.05, 0.025, 0.001, and 0.0001 per cent. In one series the chloroform was introduced previous to maceration of the brain, and in another series subsequently. In each of the twenty experiments the correct amount of chloroform, *i.e.* the percentage which had been added, was indicated by the tests.

A third series consisted in soaking twenty-three brains from rats, killed by illuminating gas, in pure chloroform for at least 24 hours.

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The brains were then washed either successively in running water, alcohol, and ether, or in running water alone, for varying periods of time. In any one group of brains which had been washed in the same way for the same length of time, closely concordant results were obtained. The amounts of chloroform present could be calculated as cc. per gm. of brain tissue, since the weight of the brain and the volume of the extract were known. For example, in Group 2, where washing in water alone was continued for 2 minutes, the amounts of chloroform were: 0.2097, 0.2059, 0.2105, and 0.2083 cc. per gm.; in Group 1, where running water was used for 10 minutes: 0.1703, 0.1721, 0.1754; and in Group 5, where water, alcohol, and ether were each used for 1 minute: 0.0978, 0.09422, 0.0912, and 0.09174 cc. per gm. It is to expected that considerable variation in the absolute amount of chloroform found in such extracts will exist, due to the differences in (1) the absorbing surfaces of the brains; (2) the method of washing; and (3) the length of time of washing. The last two causes are the most significant, and it is believed that if the volume of running water or other washing agent used, as well as the time of washing, were precisely measured and constant, the absolute amount of chloroform absorbed by a rat brain in 24 hours could be accurately determined. As a check on the reliability of the method, these experiments are satisfactory and appear to indicate that the pyridine test may safely be used to detect small amounts of chloroform in watery extracts of brains.

SUMMARY.

The pyridine reaction with chloroform and other substances containing the R-C-halogen$_3$ group in the presence of strong NaOH has been standardized as a quantitative colorimetric method for the estimation of small amounts of chloroform (0.1 down to 0.0001 per cent, corresponding to 0.01256 and 0.00001256 molar), in pure water and in aqueous extracts of animal tissues. Detailed directions for using the test are given, and several checks on the reliability of the method are described.

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DEAMINATION OF 3-AMINOHEXOSES.

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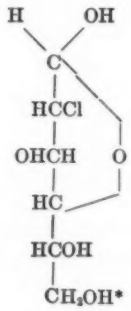
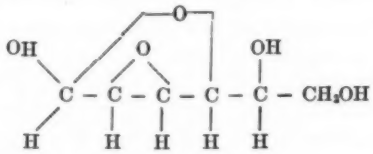
(Received for publication, October 28, 1926.)

The present paper deals with the deamination of the so called epiglucosamine of Fischer, Bergmann, and Schotte.[1] This sugar is prepared from



in which the configuration of carbon atom (2) is not known. The formation of the amino sugar through the action of ammonia passes through the intermediate stage of an ethylene oxide,

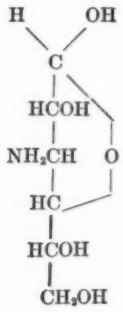


Hence, in the amino sugar, the configurations of carbon atoms

[1] Fischer, E., Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, 1920, liii, 509.

* The ring structures of the sugars of this group are not yet known. The fact that the free epiglucosamine readily forms a second internal anhydride leads one to surmise that the original ring is five-membered.

(2) and (3) are unknown. Recently Freudenberg, Burkhart, and Braun[2] prepared a new 3-aminohexose to which they assigned the following structure:



Inasmuch as this sugar was prepared from tolyl sulfodiacetone glucose, there is uncertainty of the configuration of carbon atom (3). The properties of the two amino sugars are distinctly different and the question arises whether these differences are due to dissimilarities in the configurations of only one carbon atom (2) or (3), or in those of both.

Dissimilarities in carbon atom (2) will disappear when the two sugars are converted into the corresponding osazones. Hence, if the osazones of the two sugars should show dissimilarities, the conclusion will follow that the configurations of carbon atoms (3) are not identical, and *vice versa*. The two osazones have been prepared, that of epiglucosamine by Levene and Meyer[3] and that of the new amino sugar by Freudenberg and his coworkers. The two osazones were found somewhat different. The former had a sharp melting point at 207°C.; for the second only the decomposition point is given, which is at 207°C. Often the decomposition point lies much above the melting point. The specific rotation of the first was found −41° and that of the second −58°. If the two osazones are different, then one should have a configuration

[2] Freudenberg, K., Burkhart, O., and Braun, E., *Ber. chem. Ges.*, 1926, lix, 714.

[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1923, lv, 221.

analogous to glucosazone and the other to that of allosazone. The latter two differ from one another in the following way: The glucosazone has the higher melting point and the higher levorotation. In the case of the two osazones of the amino sugars, the one with the higher rotation seems to have either the identical or a lower melting point than the isomer. It is to be regretted that Freudenberg and his coworkers, who had the two osazones in their hands, did not compare the melting points, inasmuch as the melting points of osazones depend so on the rate of heating. These authors, however, did compare their specific rotations and these may be considered correct. On the basis of the rotations, the osazone of Freudenberg and coworkers should be analogous to glucosazone and that of Levene and Meyer to allosazone. Such, indeed, was the conclusion of Freudenberg and coworkers, although they arrived at it on hypothetical grounds. However, the present assumptions may be regarded only as an indication for further and more rigorous investigation.

In the way of a beginning to such work, we decided to compare the osazones of the desamino sugars in the hope that the differences in the properties of these would be more pronounced than in the case of the amino sugars. Inasmuch as the preparation of the 3-amino sugars is very laborious, we are reporting for the present on the properties of one desamino sugar only, namely of the anhydrosugar derived from epiglucosamine, particularly since the observations on this substance have several points of fundamental importance.

Aminohexoses are now known with the amino group in position (2), (3), or (6). The first group on deamination gives rise to 2-5-anhydrosugars; the third, according to Freudenberg and Doser,[4] gives rise in the main to the parent hexoses. The behavior in this respect of the 3-aminohexoses has not yet been established. We now find that epiglucosamine forms on deamination an anhydrosugar with the ring (as seen from the model) most probably in 3-6 position. One anhydrosugar of this type has already been prepared by Fischer and Zach[5] by the action of baryta on triacetylmethylglucoside bromohydrin. In the case of Fischer and Zach

[4] Freudenberg, K., and Doser, A., *Ber. chem. Ges.*, 1923, lvi, 1243.
[5] Fischer, E., and Zach, K., *Ber. chem. Ges.*, 1912, xlv, 456.

the sugar had the configuration of an anhydroglucose, also probably with ring in position 3-6. The comparison of the osazone of the anhydrosugar of Fischer and Zach with that obtained from epiglucosamine should furnish certain indications as to the structure of the new anhydrosugar. Fischer and Zach record the melting point of their osazone at 180°C. and decomposition point at 200°C. Our osazone had a melting point of 160°C. and a decomposition point at 190°C. Fischer and Zach do not record the specific rotation of their osazone, hence the two osazones cannot be compared from that view-point. It is interesting, however, to note that glucosazone has a higher melting point than allosazone, and if there exists an analogy between the osazones of the simple sugars and those of the anhydrosugars, then the configuration of the new osazone should be that of 3-6 anhydroallosazone. For a final conclusion more evidence is required. Further work on the anhydrosugars is in progress. The results thus far obtained are published now in view of the fact that one of the authors had to discontinue his cooperation in this investigation.

EXPERIMENTAL.

The starting material was the acetate of 1-methyl-3-aminohexose previously described by Levene and Meyer. The deaminized free sugar may be obtained either by deaminizing the glucoside and subsequently hydrolyzing the anhydroglucoside, or first hydrolyzing the aminoglucoside and subsequently deaminizing the amino sugar. Only the second method will be described here in detail inasmuch as this method proved by far the most economical from the view-point of yield.

2.0 gm. of epiglucosamine acetate were dissolved in 50 cc. of 2 per cent hydrochloric acid, and the solution was boiled for 2 hours. The solution possessed a reducing power equivalent to 0.6 gm. of glucose.

For deamination the solution was cooled to 0°C., and to it an equivalent of silver nitrite was added; after 8 hours $\frac{1}{2}$ of an equivalent of silver nitrite and the corresponding amount of hydrochloric acid were added. The final solution was freed from the excess of silver ions, and it then possessed a reducing power equivalent

to 1.3 gm. of glucose. The solution was then neutralized and concentrated to a volume of 100 cc. A solution of 1.5 gm. of phenylhydrazine in glacial acetic acid was added to it and the combined solution was digested on the water bath for 10 minutes. A very voluminous, gelatinous, orange-yellow precipitate formed which consisted of microscopic rosettes. To the filtrate from the first crop of the osazone was added a second portion of 1.0 gm. of phenylhydrazine dissolved in glacial acetic acid. The solution was allowed to digest 10 minutes and the second crop of osazone, which had the same appearance as the first, was filtered off and dried.

The two crops of osazone were combined and recrystallized from water containing a little pyridine. The material prepared in this manner, on heating in a capillary tube, contracted at 120°C., melted at 160°C., and decomposed at 190°C. The recrystallization from pyridine and water was repeated and the product behaved on heating in the same manner as before recrystallization. The osazone was then recrystallized from dilute alcohol, from which solvent it deposited in bright yellow microscopic rosettes. It melted sharply at 160°C. and decomposed at 185–190°C.

0.0456 gm. substance: 0.1040 gm. CO_2 and 0.0242 gm. H_2O.
0.0516 " " : 7.40 cc. N_2 at 25°C. and 764.0 mm.

$C_{18}H_{20}O_2N_4$ (340.3). Calculated. C 63.49, H 5.93, N 16.47.
Found. " 63.50, " 6.11, " 16.51.

Two samples were used for the measurement of the optical activity. One sample was obtained by recrystallization from pyridine and water. It rotated in methyl alcoholic solution as follows:

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.22° \times 100}{0.500 \times 1} = -44°. \qquad [\alpha]_D^{20} = \frac{-0.04° \times 100}{0.500 \times 1} = -8°.$$

The second sample was obtained by further recrystallization of the first sample from dilute alcohol. Its rotation in methyl alcoholic solution was the following.

Initial. Equilibrium.

$$[\alpha]_D^{20} = \frac{-0.12° \times 100}{0.500 \times 1} = -24°. \qquad [\alpha]_D^{20} = \frac{-0.04° \times 100}{0.500 \times 1} = -8°.$$

XUM

GLASS SCREENS FOR THE TRANSMISSION OF THE LIGHT RADIATIONS CURATIVE OF RICKETS.

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(Received for publication, October 18, 1926.)

The following preliminary experiments form part of a more detailed study (to be published later) of the effect of light of varying wave-length on the development of rickets in the rat.

A study such as this involves the use of filters, and in the two experiments described in this paper trials were made of three filters to test their efficiency for the transmission of antirachitic radiations.

General Method of Experiment.

Rats were fed on a standard rickets-producing diet and exposed daily to sunlight transmitted through these filters.

Filters.

The three filters used,[1] manufactured by the Corning Glass Company, were:

1. No. G86B, 4.3 mm. thick, a neutral filter showing spectrographically a transmission to 296 $\mu\mu$ in the ultra-violet, and by spectrophotometric measurement a weak transmission of 0.5 per cent light of wave-length between 300 and 305 $\mu\mu$.

2. No. G980A, 4.98 mm. thick, a white glass filter showing spectrographically a transmission to 240 $\mu\mu$ in the ultra-violet, and by spectrophotometric measurement a transmission of 80 per cent light of wave-length 300 $\mu\mu$.

3. No. G986A, 3 mm. thick, a purple glass filter, opaque to visible light of wave-length longer than 425 $\mu\mu$, showing spectro-

* Medical Travelling Fellow of the Rockefeller Foundation.

[1] Hood, H. P., *Science*, 1926, lxiv, 281.

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graphically a transmission to 265 $\mu\mu$ in the ultra-violet, and by spectrophotometric measurement a transmission of 77 per cent light of wave-length 300 $\mu\mu$.

Experiment I.

Rats bred at the Albino Supply Company, Philadelphia, were used. They were all 31 days old and weighed approximately 45 gm. at the beginning of the experiment. They were divided into three groups of five rats in each group. Each group contained representatives of the same family and sex. Each rat was kept in a separate cage and all were fed on Steenbock's modification of

TABLE I.

Rats Fed 28 Days on Steenbock's Modification of McCollum's Diet 3143.

Group I. Exposed to full sunlight.
Group II. Exposed to sunlight through filter No. G86B.
Group III. Control group kept in complete darkness.
Per cent Ca figures = average value of five rats in each group.

Group.	In bones. Per cent Ca estimated on dry weight of bone.	In blood. Mg. per 100 cc. serum.		Histological findings.
		Ca	P	
I	16.0	10.4	5.6	No evidence of rickets.
II	13.9	9.9	3.2	All showed definite rickets.
III	14.5	9.9	3.3	" " " "

McCollum's Diet 3143 (1) (corn 76 per cent, wheat gluten 20 per cent, $CaCO_3$ 3 per cent, NaCl 1 per cent).

Group I was exposed to sunlight filtered through the glass G86B in a house at the Boyce Thompson Institute roofed in with this filter. Group II was exposed to full sunlight in open cages placed outside this glass-roofed house. Group III was kept in complete darkness.

The experiment was conducted in May and June, 1925, which proved to be a period of intense sunlight. The irradiated rats received maximum exposures to sunlight, on an average 6 to 7 hours daily.

At the end of 28 days they were bled under light ether anesthesia, and the pooled blood serum in each group was analyzed for Ca by the Kramer-Tisdall (2) method and for P by the Briggs (3) modification of the Bell-Doisy method.

The bones of both hind legs were analyzed for Ca,[1] and a histological examination was made of the bones of the forearm. The result of this experiment is set out in Table I.

Table I shows that rats irradiated for 6 to 7 hours daily with sunlight filtered through glass G86B for 28 days failed to receive protection. This finding confirms that of Hess (4) (1923) working with this filter. Those rats, on the other hand, exposed to the same sunlight in open cages were completely protected.

TABLE II.

Rats Fed for 28 Days on Steenbock's Modification of McCollum's Diet 3143.

Group I. Exposed to full sunlight.

Group II. Exposed to sunlight through filter G986A (ultra-violet light only).

Group III. Exposed to sunlight through filter G980A (visible and ultra-violet light).

Group IV. Control group kept in complete darkness.

Per cent Ca figures = average value of five rats in each group.

Group.	In bones. Per cent Ca estimated on dry weight of bone.	In blood. Mg. per 100 cc. serum.		Histological findings.
		Ca	P	
I	15.8	12.6	6.4	No evidence of rickets.
II	15.9	12.5	6.7	" " " "
III	15.7	12.4	6.5	" " " "
IV	13.5	9.6	4.9	All showed definite rickets.

Experiment II.

In the fall of 1925 a trial was made of the two filters G980A and G986A described above. The general technique of this experiment was identical with that described in Experiment I. As, however, only small quantities of the filters were available, the

[1]Calcium was determined on the bones, after extraction of the fat, first with alcohol, followed by ether. The fat-free bones were ashed in porcelain at red heat until all organic matter had disappeared. They were then dissolved in nitric acid, made up to a certain volume, and an aliquot taken for Ca determination. The solution was neutralized with ammonium hydroxide, using methyl red as an indicator, precipitated with saturated ammonium oxalate, washed, and titrated in the usual way with 0.01 potassium permanganate.

rats were exposed to sunlight in specially designed irradiation cages covered on roof and sides with the two filters. The results of this experiment are set out in Table II.

Table II shows that the degree of protection obtained by the rats from sunlight filtered through the glasses G980A and G986A was identical with that obtained by those animals which were exposed in open cages to full sunlight.

The efficiency of these filters for the transmission of antirachitic radiations has been abundantly confirmed in more recent experiments using minimum exposures to light from a carbon arc lamp. A full report and discussion will be published later.

In conclusion my thanks are due to Dr. E. A. Park for much stimulating advice and helpful cooperation, to Dr. Crocker of the Boyce Thompson Institute for the hospitality of the Institute where these experiments were carried out, and to Dr. Mees of the Eastman Kodak Company for kindly supplying me with spectrophotometric measurements of the filters used.

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METHODS FOR THE DETERMINATION OF SOME OF THE NON-PROTEIN SULFUR COMPOUNDS OF BLOOD.

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(Received for publication, October 22, 1926.)

In a paper published in 1921 (1) there was described a micro method for the determination of inorganic sulfates in blood. By means of this method numerous observations were made of the content of inorganic sulfates of normal and pathological human blood and of the blood of several species of animals. As a result of this work it was found that normal human blood has a sulfate content of 0.5 to 1.0 mg. of sulfur from 100 cc. No evidence could be obtained of the presence in blood of sulfur in ethereal combination, although the fact was recognized that the evidence presented on this point was inconclusive.

At the time the above paper was written we were able, after a careful search of the literature, to find only one paper dealing with the sulfate content of blood (2). In this publication De Boer presents the results obtained on the determination of inorganic sulfates in horse blood made by a sedimentation method after the removal of proteins by ultrafiltration, which fraction he found to be 0.02123 per cent calculated as SO_4 (or 7 mg. of S per 100 cc.). Since this time a number of communications have appeared which deal with the non-protein sulfur compounds of the blood.

Heubner and Meyer-Bisch (3) have called attention to the early work of Gurber (4) who found 18 to 25 mg. of dialyzable sulfate (as H_2SO_4) in horse serum. These investigators report the results of dialysis experiments carried on for 14 days on the serum of a patient suffering from nephritis and albuminuric retinitis. This material showed a content of 24 mg. of H_2SO_4 per 100 cc. They state that when an attempt was made to deproteinize serum by the usual methods (95 per cent alcohol, heat and acetic acid, etc.) practically all of the inorganic sulfate is absorbed by the coagulum.

Recently Browinski (5) has published the results of his observations on the sulfur compounds of horse and of beef serum. The determinations were made by the usual gravimetric method after removal of protein by means

of trichloroacetic acid. This author was unable to detect the presence of ethereal sulfates, but believes that he has demonstrated the presence of neutral sulfur compounds. In a series of determinations of the serum of the horse and of the ox he reports for inorganic sulfates figures varying from 9.55 to 12.0 mg. of S per 100 cc. of serum, and finds that neutral sulfur constitutes on an average 50 per cent of the total non-protein sulfur.

In a second communication Meyer-Bisch (6) has presented a series of results for the total H_2SO_4 (inorganic plus ethereal sulfates) of the serum of patients suffering from a variety of pathological conditions. These determinations were made by the gravimetric method on the dialysate from oxalated blood. For normal specimens 14 to 17 mg. of total SO_4 per 100 cc. of serum were found, while in a series of determinations made on pathological material figures varying from 9 mg. in a case of polycythemia to 49 mg. in one of lumbago are reported. In eight nephritic sera the figures vary from 19 to 72 mg.

Another recent contribution to the study of non-protein sulfur of blood has been made by Kahn and Postmontier (7). These investigators worked with oxalated blood deproteinized by means of trichloroacetic acid. In the blood of four fracture and five hernia cases (which may be assumed to represent normal material) the total sulfur varies from 2.5 to 3.8 mg. of SO_4 per 100 cc. of blood.

One other procedure has been proposed by White (8), but as this method was devised for examination of the blood of animals which had received large injections of sulfates, and not for the analysis of normal blood, it need not be considered here.

If, in order to obtain uniformity of notation all values are recalculated, where necessary, to the basis of mg. of sulfur per 100 cc., the following summary presents the results now available on the non-protein sulfur constituents of blood and serum:

Inorganic Sulfates.

Gurber (1894), 6 to 8 mg. of S per 100 cc. in horse blood.

Heubner and Meyer-Bisch (1921), 8 mg. of S per 100 cc. in human serum.

Denis (1921), 0.5 to 1.0 mg. of S per 100 cc. in normal citrated human blood; increased values up to 16.0 mg. in nephritis. 1.8 to 4.0 mg. in the blood of various animals (beef, horse, sheep, etc.).

Browinski (1924), 9.55 to 12.0 mg. of S per 100 cc. in horse serum.

Meyer-Bisch (1924), 4.8 to 5.8 mg. of S per 100 cc. in human oxalated plasma; 6.4 to 24.0 mg. in nephritic plasma. These figures represent inorganic plus ethereal sulfates.

Kahn and Postmontier (1925), 0.6 to 1.0 mg. of S per 100 cc. in human oxalated blood.

The following summarizes the available data regarding the presence in blood of ethereal sulfates or "neutral" sulfur compounds:

Heubner and Meyer-Bisch (1921) believe that ethereal sulfates exist in blood but give no figures for this fraction.

Denis (1921) was unable to obtain proof of the presence in blood of these bodies.

Browinski (1924) cites experiments which in his opinion prove the absence of ethereal sulfates in horse blood. On oxidation of the trichloroacetic acid filtrate by means of sodium peroxide he obtained evidence of the presence of considerable quantities of neutral sulfur in amounts varying from 44.13 to 56.98 per cent of the total sulfur.

Heubner believes that ethereal sulfates are present in blood but makes no sulfate determinations for the fraction as the figures presented by him are for "total sulfates."

Kahn and Postmontier apparently consider that three forms of non-protein sulfur (inorganic, ethereal, and neutral) exist in blood, and the figures given for neutral sulfur (total non-protein sulfur minus total sulfates) amount to from one-fourth to one-sixth of the values for total sulfates.

From the above discussion it is clear that, in view of the great diversity of results obtained by different investigators, there is need for further work in this field.

The work, the results of which will be reported below, has been divided into three parts. First a comparison was made of the results obtained for the inorganic sulfate fraction, when dialysis and when various methods of protein precipitation were employed; second a series of experiments was carried out to determine whether a fraction of the non-protein sulfur of blood exists in the form of the ethereal sulfates or of neutral sulfur; third an attempt has been made to devise micro methods for the quantitative determination of these forms of sulfur.

In the reports on blood sulfates so far published the question of the effect of the anticoagulant used on the purity of the barium sulfate precipitate seems in most cases to have escaped critical consideration. Many of the determinations have of course been carried out on serum, but on the other hand several investigators (Heubner, Kahn) have used oxalated blood or the plasma therefrom, while others (White, Denis) have employed citrated blood exclusively. As the solubility of barium citrate is approximately four times greater than that of barium oxalate (9) (0.0406 gm. of the former and 0.0105 gm. of the latter salt being soluble in 100 cc. of water at 18°C.), the use of citrate as an anticoagulant would appear to be theoretically, at least, a safer procedure.

XUM

Dialysis Experiments.

100 to 200 cc. of citrated whole blood were shaken with 5 cc. of toluene and 30 mg. of thymol, and were then placed in a Schleicher and Schüll dialyzing cylinder which was suspended in a beaker containing 1 liter of distilled water. Dialysis was allowed to continue for several days at room temperature, and sometimes an additional period of 3 days. The water in the dialyzer was changed at intervals of 12 hours. At the end of the experiment the various portions of dialysate were acidified with hydrochloric acid, evaporated to small volume, the sulfate precipitated by means of barium chloride, filtered, ignited, and weighed in the usual manner.

In some of the later experiments Schleicher and Schüll folded parchment dialyzers were used in place of dialyzing cylinders. The results of several experiments of this type are presented below.

Experiment 1.—100 cc. of arterial dog blood, taken under ether anesthesia, which contained 0.25 gm. of potassium oxalate and 5 cc. of toluene were dialyzed for 3 days. Sulfate in the total dialysate amounted to 7.8 mg. of S per 100 cc. of blood. After 4 additional days dialysis there was obtained 0.28 mg. of S, or a total after 7 days dialysis of 8.08 mg.

Experiment 2.—100 cc. of blood from the same animal containing 0.5 gm. of sodium citrate and 5 cc. of toluene gave after 3 days dialysis 8.2 mg. of S, and after 4 additional days 0.18 mg., making a total of 8.38 mg. of S per 100 cc. of blood.

Experiment 3.—Two dialysis experiments made on oxalated, and seven on citrated beef blood, carried out on material taken from eight animals, gave results varying from 7.7 to 9.6 mg. of S per 100 cc. of blood.

Experiments with Several Protein Precipitants.

In these experiments 100 cc. portions of blood were precipitated as indicated below, and the sulfate content of the protein-free filtrate determined gravimetrically by means of the barium chloride precipitation. As protein precipitants we have used picric and hydrochloric acids, mercuric chloride and hydrochloric acid (1), acetic acid and heat, and trichloroacetic acid.

The first three methods were soon abandoned as it was found

impossible when using these methods of precipitation to recover quantitatively sulfate added to blood. In the case of the picric acid, the high results obtained were later found to be due to the fact that it is extremely difficult to obtain even by repeated recrystallization, samples of this substance free from minute traces of sulfates; the acetic acid precipitation proved extremely laborious, and, as is well known, invariably furnished a filtrate containing minute quantities of protein which interfered with the subsequent quantitative precipitation of the barium sulfate, while results obtained by the mercuric chloride precipitation were invariably low. The precipitant of choice appeared therefore to be trichloroacetic acid, by means of which we find it possible to obtain excellent results on the recovery of sulfates added to blood and to plasma.

Early in the course of the work it was found however that every sample of C.P. trichloroacetic acid purchased contained traces of sulfates; this impurity is however readily removed by redistilling the acid *in vacuo* at a pressure of 15 to 20 mm.

The gravimetric procedure which we have used as a basis of our determination of the recovery of sulfates added to blood, and also as a check on the nephelometric procedure described below is as follows: 50 cc. of citrated blood or plasma are treated with 110 cc. of distilled water and 40 cc. of a 20 per cent solution of trichloroacetic acid. The mixture is thoroughly shaken, allowed to stand for 15 minutes, the precipitate centrifuged off, and the supernatant liquid filtered through acid-washed filter paper (Whatman's No. 40).

The non-protein sulfur fractions are then determined as follows:

Inorganic SO_4.—To 100 cc. of the filtrate are added 100 cc. of distilled water and 5 cc. of 5 per cent barium chloride solution. This mixture is allowed to stand for at least 12 hours at room temperature, and the precipitated barium sulfate is then filtered on a tared Gooch crucible provided with an asbestos mat, washed, dried, and weighed in the usual manner.

Total SO_4.—To 100 cc. of the filtrate are added 100 cc. of normal hydrochloric acid, and the mixture is heated for 2 hours on an electric plate, with the occasional addition of distilled water should the volume decrease too much. At the end of this period 5 cc. of 5 per cent barium chloride are added and after standing overnight the precipitate is filtered off.

XUM

Ethereal Sulfates.—These are calculated by difference from the figures obtained for inorganic and for total sulfates.

Total Sulfur.—To 50 cc. of blood filtrate there are added 5 cc. of Benedict's (10) copper nitrate reagent and the mixture is evaporated to dryness on a hot plate, ignited with a free flame until no more fumes are evolved, dissolved in 20 cc. of 10 per cent hydrochloric acid, and after the addition of 100 cc. of distilled water precipitated with 5 cc. of 5 per cent barium chloride solution. After standing for several hours the precipitated barium sulfate is then filtered off on a Gooch crucible, washed, ignited, and weighed.

TABLE I.

Total Sulfates in Beef Blood and Plasma Determined by Dialysis and by Precipitation with Trichloracetic Acid.

Sample.	Total SO_4 as mg. of S per 100 cc. of blood or plasma.	
	Determined by dialysis.	Determined by precipitation.
Beef.		
Blood 1	7.7	7.6
" 2	8.0	7.8
" 3	7.7	7.9
Dog.		
Blood 1	8.2	8.3
Beef.		
Plasma 1	6.3	6.4
" 2	6.8	6.8
" 3	7.0	7.4

Neutral Sulfur.—This is calculated by difference from the figures obtained for total S and total SO_4.

The methods described above are of course merely adaptations of the technique in use for many years with urine; the chief points requiring experimental proof are therefore the question as to whether sulfates in blood can be quantitatively extracted by means of trichloroacetic acid, and whether the method of extraction leaves any substances in the filtrate which would interfere with the subsequent precipitation of the sulfate by barium chloride.

In Table I we have collected the results on total sulfates obtained by dialyzing citrated beef blood and plasma for 7 days, and also the figures for the same fraction when total sulfates were

determined in the filtrate after removal of proteins with trichloroacetic acid.

Table II contains the results obtained in a series of experiments in which citrated beef blood or serum which had been dialyzed against distilled water (in the presence of toluene and thymol) for 7 days, or until the dialysate gave on concentration no qualitative test for chlorides or sulfates, was precipitated with trichloroacetic acid after the addition of known amounts of sulfates, and this radicle determined in the filtrate.

TABLE II.

Results on the Recovery of Sulfate Added to Blood and Plasma Previously Dialyzed Free of Sulfates and Chlorides.

Experiment No.	Material.	K_2SO_4 added as mg. of S per 100 cc.	SO_4 recovered as mg. of S per 100 cc.	Recovery.
				per cent
1	Blood.	1	0.93	93
2	"	2	2.06	103
3	"	2	2.00	100
4	"	3	3.10	103
5	"	3	3.08	102
6	"	4	3.88	97
7	"	4	3.86	96
8	"	5	5.06	101
9	"	5	5.08	101
10	"			
11	Plasma.	5	5.10	102
12	"	5	4.98	99

As, during dialysis, the blood and plasma had increased greatly in volume, water was added in such amounts that 150 cc. of the dialyzed material were equivalent to 50 cc. of the original blood or plasma. The precipitations were carried out as follows: to 150 cc. of the dialyzed and diluted material were added 10 cc. of a potassium sulfate solution of known concentration and 40 cc. of 20 per cent trichloroacetic acid. Subsequent treatment was identical with the procedure described above.

The results presented in Table II indicate that, contrary to the statements of Heubner and Meyer-Bisch (3), it is possible to recover sulfates from blood by precipitation methods with a fair degree of accuracy.

Micro Method for Determination of SO_4.

The macro methods described above are of course of limited usefulness on account of the large amount of blood required and the time-consuming nature of the gravimetric technique involved. We have therefore made an attempt to devise micro methods which could be used on small amounts of blood, and which would be of greater accuracy than the original procedure proposed for this purpose by Denis (1).

By the nephelometric procedure outlined below it is possible to determine inorganic sulfates, total sulfates, and total sulfur in the protein-free filtrate obtained from 30 cc. of human blood or from 15 cc. of dog or beef blood, with a maximum error of 5 per cent.

Solutions Required.—20 per cent trichloroacetic acid solution prepared from sulfate-free redistilled trichloroacetic acid. It is usually necessary to filter this through sulfate-free filter paper, as it usually contains suspended particles. 1.0 per cent barium chloride solution. 0.6 N NaOH solution. We have found it necessary to prepare this solution from metallic sodium as all the specimens of sodium hydroxide we were able to secure contained traces of sulfates. 0.1 N hydrochloric acid. Normal hydrochloric acid. 5 per cent gelatin solutions (sulfate-free). All the specimens of high grade commercial gelatin contain traces of sulfates, and it is therefore necessary to free the gelatin from this impurity before its use as a protective colloid.

Sulfate-free solutions that will keep for long periods without the formation of mould, etc., may be prepared as follows: To 50 gm. of any high grade commercial gelatin[1] add 900 cc. of approximately 0.01 N hydrochloric acid and 100 cc. of a 5 per cent solution of barium chloride. Immerse the flask in a bath of boiling water and heat at this temperature, with occasional shaking, for 1 hour. At the end of this period of heating remove the flask from the water bath, cool in running water, add 50 cc. of egg white, mix thoroughly, and heat again in a boiling water bath for about 30 minutes or until the egg white has coagulated and carried down all traces of colloidal barium sulfate. Centrifuge while hot, using 50 or 100 cc. centrifuge tubes, pour off the clear supernatant liquid

[1] For most of our work we have used the Difco brand of powdered gelatin manufactured by the Digestive Ferments Company of Detroit.

into small wide mouth bottles of about 30 cc. capacity, stopper these bottles with cotton, sterilize the preparation by heating the bottles for 1 hour in boiling water, allow to cool, and store in the refrigerator. When kept in an ordinary domestic refrigerator our preparations have remained in good condition for as long as 8 months.

Zinc Nitrate Oxidizing Mixture.—Benedict's copper nitrate oxidizing mixture, which was originally suggested for the determination of total sulfur in urine, has since been applied with satisfactory results to many types of biological material. In attempting to use the Benedict reagent in this work it was found that the

TABLE III.

Comparative Results Obtained in Determination of Total Sulfur by Use of Copper Nitrate and Zinc Nitrate Oxidizing Reagents.

Results expressed in mg. of S per 100 cc. of blood.

Dog blood.	Gravimetric determination with Benedict's reagent.	Nephelometric method.		Per cent error on basis of gravimetric method.	
		$Cu(NO_3)_2$	$Zn(NO_3)_2$	$Cu(NO_3)_2$	$Zn(NO_3)_2$
1	10.528	11.00	10.56	+4	+0.4
2	9.968	10.66	9.81	+7	+1.0
3	10.592	10.98	10.26	+4	−3
4	10.304	11.74	9.50	+13	−8
5	8.736	9.42	9.29	+8	+6
6	8.40	14.70	8.81	+75	+5
7	8.74	11.00	8.50	+26	−2
8	9.74	11.08	9.64	+13	−1

blue color of the copper salts interfered with the nephelometric reading of the suspensions of barium sulfate and we have therefore found it necessary to prepare an oxidizing mixture in which copper is replaced by zinc nitrate.

This reagent is prepared as follows: 25 gm. of zinc nitrate, C.P.; 25 gm. of sodium chloride, C.P.; 10 gm. of ammonium chloride, C.P.; 100 cc. of distilled water. When the salts are completely dissolved, the mixture is filtered through an ashless filter paper. Each lot of reagent should of course be tested for the presence of sulfates before use. Comparative results obtained with this reagent and with the Benedict reagent are given in Table III.

Standard K_2SO_4 Solutions.—Two standards are required. Standard 1 contains 0.5437 gm. of potassium sulfate per liter. 1 cc. of this solution is equivalent to 0.1 mg. of S. Standard 2 is prepared by diluting 1 volume of Standard 1 with an equal volume of water. 1 cc. of this solution is equivalent to 0.05 mg. of S.

TABLE IV.

Comparison of Results Obtained by Gravimetric and Nephelometric Determinations of the Sulfur Compounds in Blood.

Sample.	Gravimetric method. Mg. of S per 100 cc. of blood.			Nephelometric method. Mg. of S per 100 cc. of blood.			Per cent error on basis of gravimetric method.		
	Inorganic sulfates.	Total sulfates.	Total sulfur.	Inorganic sulfates.	Total sulfates.	Total sulfur.	Inorganic sulfates.	Total sulfates.	Total sulfur, Zn method.
Dog.									
Blood 1...	2.912		10.528	2.84		10.56	−3		+4
" 2...	4.032		10.592	3.86		10.26	−4		−3
" 3...	2.52		8.736	2.4		9.42	−4		+7
" 4...	3.25		8.96	3.04		9.53	−6		+5
" 5...	2.464		8.736	2.6		8.50	+4		−2
" 6...	3.416		8.40	3.2		8.81	−6		+5
" 7...	3.864		9.74	4.00		9.64	+3		−1
" 8...	2.408		9.968	2.23		9.81	−7		−1
Human.									
Blood 1...	1.02	2.006	4.51	1.22	2.00	4.26	−8	0	−5
" 2...	0.35	1.89	3.52	0.348	1.69	3.75	0	−10	+6
" 3...	1.72	3.36	5.53	1.56	3.20	5.21	−10	−5	−4

Nephelometric Determination of Inorganic Sulfates in Blood Serum or Plasma.

The blood serum or plasma is deproteinized as described above under the discussion of the gravimetric method.

With dog or beef blood proceed as follows: 15 cc. of trichloroacetic acid filtrate, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, 5 cc. of 1 per cent barium chloride solution. Allow to stand for 15 minutes and then read in the nephelometer against the following standard which has been prepared at the same time as the unknown. 1 cc. of standard K_2SO_4 solution equivalent to 0.1 mg. of S., 14 cc. of 0.1 N HCl, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, 5 cc. of 1 per cent barium chloride.

Determination of Inorganic Sulfate in Human Blood.

On account of the very small concentration of inorganic sulfate in normal human blood it is necessary to use 25 cc. of trichloroacetic acid blood filtrate for the determination. To this add 4 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 1 cc. of 5 per cent $BaCl_2$. Allow to stand 15 minutes before reading in the nephelometer.

The standard is made with 1 cc. of K_2SO_4 containing 0.05 mg. of S per cc., 25 cc. of 0.1 N HCl, 3 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 1 cc. of 5 per cent $BaCl_2$. Allow standard and unknown to stand 15 minutes before reading in the nephelometer.

In nephritic blood where the sulfate content is high the procedure outlined for dog or beef blood may be used.

Total Sulfates (in Dog or Beef Blood).

To 10 cc. of blood filtrate add 4 cc. of normal HCl and evaporate at such a rate that solid particles begin to settle out in not less than 15 to 20 minutes. At this point the heating should be discontinued. This evaporation is best carried out in a large (20 × 200 mm.) Pyrex test-tube heated by means of a micro burner. The evaporation of the last few cc. of liquid must of course be carried out with considerable care in order to prevent undue discoloration. To the residue in the test-tube add 15 cc. of water, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride solution. Allow to stand 15 minutes and then read against a standard which has been prepared at the same time as the unknown in the following manner: 1 cc. of standard K_2SO_4 solution (equivalent to 0.1 mg. of S), 4 cc. of normal HCl. Evaporate in a manner identical with that used for the unknown; to the residue add 15 cc. of water, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride solution.

Total Sulfates in Human Blood.

The method for human blood is identical with that for dog or beef blood with one exception; namely, that it is necessary to evaporate 15 cc. of filtrate with 4 cc. of the N HCl rather than 10 cc. of filtrate. It is best to prepare two standards, the 0.05 mg. of S standard, and the 0.1 mg. of S standard.

XUM

If nephritic blood, having a high sulfur content, is used, the method for dog or beef blood may be used unaltered.

Total Sulfur in Dog or Beef Blood.

To 3 to 5 cc. of blood filtrate add 1 cc. of zinc nitrate oxidizing mixture. Evaporate to dryness in a large (20 × 200 mm.) Pyrex tube and continue to heat with a free flame until no more fumes are given off; dissolve the residue in 2 cc. of normal HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin solution, and 5 cc. of 1 per cent barium chloride; allow to stand 15 minutes, then read

TABLE V.

Non-Protein Sulfur Compounds in the Blood of Various Animals.

Source of blood.	Mg. of S per 100 cc. of blood.					Per cent on basis of total sulfur.			
	Inorganic sulfates.	Total sulfates.	Ethereal sulfates.	Total sulfur.	Neutral sulfur.	Inorganic sulfates.	Total sulfates	Ethereal sulfates.	Neutral sulfur.
Human.....	1.12	2.006	0.886	4.51	2.504	24.8	44.4	19.6	55.5
"	0.35	1.89	1.54	3.75	2.21	9.32	50.6	41.1	59.0
Dog.....	3.64	8.29	4.65	11.87	3.58	30.6	69.8	39.1	30.1
"	2.02	6.61	4.59	10.08	3.47	20.0	65.5	45.5	34.1
"	3.08	6.16	3.08	8.736	2.576	35.2	69.4	35.2	30.6
Beef.....	2.35	5.624	3.274	7.29	1.666	32.2	77.0	42.2	22.8
"	2.576	6.176	3.6	8.59	2.412	29.5	71.8	41.9	28.1
Goat.	11.28	16.88	5.6	23.96	7.08	47.2	70.7	23.4	29.6
"	6.48	7.156	0.672	12.852	5.696	50.7	55.9	5.25	44.5
Rabbit.....	0.533	2.54	2.007	7.50	4.96	7.11	33.9	2.67	66.1

in the nephelometer against a standard prepared as follows and as nearly as possible at the same time as the unknown: 1 cc. of K_2SO_4 solution (equivalent to 0.1 mg. of S), 1 cc. of oxidizing mixture. Evaporate to dryness and heat with a free flame by the same technique used for the unknown, then dissolve the residue in 2 cc. of normal HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin, and 5 cc. 1 per cent barium chloride.

Total Sulfur in Human Blood.

The method for total sulfur in human blood varies from that for dog or beef blood only in the amount of blood filtrate oxidized with the $Zn(NO_3)_2$ oxidization mixture. It is generally necessary

to use from 5 to 10 cc. of filtrate with normal blood; as little as 3 cc. of filtrate may be used in blood showing sulfur retention.

Ethereal sulfates are calculated by difference between the values obtained for total and inorganic sulfates; neutral sulfur by difference from the values obtained for total sulfur and total sulfates.

DISCUSSION.

The quantitative precipitation of colloidal barium in uniform suspension is only accomplished under certain conditions, and in order to bring about this result, a protective colloid has been used; namely, 1 cc. of 5 per cent gelatin in 25 cc. volume. Satisfactory colloidal precipitation will not occur under these conditions if the amount of sulfur in 25 cc. volume is more than 0.16 to 0.2 mg. of S or less than 0.02 mg. of S. If more than 0.20 mg. of S be present, the precipitated particles are larger than those of a true colloidal sol and soon settle out making the suspension uneven; if smaller, the cloud is too light to read in the nephelometer. Since the amount of sulfur present as sulfate determines to some extent the size of the particles, it is best to prepare both the 0.1 mg. and the 0.05 mg. of S standard and read the unknown against the standard which appears most nearly to approach it in turbidity.

In precipitating barium sulfate in the presence of other salts it is necessary to take into consideration the fact that barium phosphate is also insoluble in slightly acid, neutral, or alkaline solution. The solution must then be acid. If however the solution be too acid, barium acid sulfate, a soluble salt, is formed and the precipitation of the sulfate ion is incomplete. From long experimental practice it has been found that a pH of 3.0 to 3.8 is optimum for the precipitation of all the barium sulfate and none of the barium phosphate. Sometimes in work on pathological material the trichloroacetic acid filtrates are of stronger acidity than usual, due apparently to the presence of abnormally small amounts of blood proteins. In this case the directions given for the amount of sodium hydroxide to produce the correct pH may have to be varied. If upon precipitation of a filtrate no sulfate shows as a cloud, before deciding that none is present it is advisable to determine the pH. It has long been known that certain ions have a tendency to prevent quantitative sulfate precipitation and that the presence of others is beneficial to this process. We have confirmed this fact

in that we have found that the presence of sodium chloride is essential to obtain a correct nephelometric reading.

In all the determinations the neutralization of hydrochloric acid by sodium hydroxide has been used as the source of the necessary sodium chloride with the exception of the total sulfur, in which case sodium chloride is present in the oxidation mixture.

If the oxidation mixture used in the determination of total sulfur is not heated until all trace of nitrate is decomposed and driven off, very low results will be obtained; indeed, in some cases the deficiency may amount to as much as 50 per cent.

SUMMARY.

Nephelometric micro methods for the quantitative determination of inorganic, total sulfates, and total non-protein sulfur in blood are described, and analyses are presented showing the distribution of these sulfur compounds in human blood and in the blood of several species of animals.

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NEPHELOMETRIC METHODS FOR THE DETERMINATION OF SOME SULFUR COMPOUNDS IN URINE.

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The nephelometric methods for the determination of the non-protein sulfur compounds of blood described in the preceding paper may also be used with urine. There are now available several excellent methods for the determination of these constituents in urine, for in addition to the standard gravimetric technique various modifications of the benzidine titration method have been proposed, and in addition Lorber (1) has recently suggested a nephelometric procedure. The method described below possesses, we believe, certain qualities which make it of value in cases where extremely small quantities of urine are available for analysis either on account of the small size of the experimental animal, or because of the necessity of making analysis of urine collected for relatively short periods. It also possesses the advantage of requiring for its use the minimum of time and of reagents. The details of the application of this method to the determination of the various sulfur fractions in urine are as follows.

Solutions and Apparatus.

The reagents required are identical with those used on the work in blood. As the necessary details regarding the preparation of these solutions are given in the preceding paper they will not be repeated here.

All readings have been made with the Kober nephelometer.

Dilution of Urine.

As our nephelometric procedure for the determination of sulfates gives the best results when concentration of the sulfate ion is

in the neighborhood of 0.1 mg. of S, it is invariably necessary to dilute the urine. We have found that the most convenient dilutions are 1 to 2 or 1 to 5 for human urine and 1 to 10 for the urine of cats and dogs.

We have found it most convenient in practice to dilute the urine to a proper concentration for the total sulfur determination, and if necessary to use 2 cc. of this dilution in place of 1 cc., as recommended below for the determination of inorganic and total sulfates. This additional volume must of course be balanced by the addition of 1 cc. more water to the standard.

Determination of Inorganic Sulfates.

To 1 cc. of the dilute urine (which contains in the neighborhood of 0.1 mg. of S as SO_4) add 15 cc. of 0.1 N HCl, 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent sulfate-free gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution. Allow to stand 15 minutes and read in the nephelometer against a standard which has been prepared at the same time as the unknown, and in which 1 cc. of diluted urine is replaced by 1 cc. of K_2SO_4 standard equivalent to 0.1 mg. of S.

Determination of Total Sulfates.

Place 1 cc. of diluted urine in a Pyrex test-tube together with 4 cc. of 1 N HCl, and boil gently with a free flame for not less than 15 to 20 minutes. When solid particles begin to settle out, heating should be discontinued, and the residue dissolved in 15 cc. of 0.1 N HCl. To this solution then add 2 cc. of 0.6 N NaOH, 1 cc. of 5 per cent gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution. The standard is prepared at the same time by substituting for the diluted urine 1 cc. of K_2SO_4 solution equivalent to 0.1 mg. of S. Both standard and unknown are allowed to stand for 15 minutes and are then read in the nephelometer.

Determination of Ethereal Sulfates.

This fraction is calculated in the customary manner by difference between the values for total sulfates and inorganic sulfates.

Determination of Total Sulfur.

Place 1 cc. of diluted urine in a Pyrex test-tube, add 1 cc. of the $Zn(NO_3)_2$ oxidation mixture, and evaporate to dryness over a free flame. Dissolve the residue in 2 cc. of 1 N HCl, add 15 cc. of distilled water, 1 cc. of 5 per cent gelatin, and 5 cc. of 1 per cent $BaCl_2$ solution.

TABLE I.

Comparison of Results Obtained on Urine by Gravimetric and Nephelometric Methods.

Urine.	Inorganic sulfates.			Total sulfates.			Total sulfur.		
	Gravimetric.	Nephelometric.	Per cent error.	Gravimetric.	Nephelometric.	Per cent error.	Gravimetric.	Nephelometric.	Per cent error.
I, human.	0.465	0.440	−5	0.504	0.504	0	0.604	0.644	+6
II, "	0.190	0.186	−2	0.22	0.217	−1.3	0.250	0.251	+0.4
III, "	0.325	0.319	−1.8	0.369	0.376	+2	0.408	0.426	+2.6
IV, "	0.129	0.1294	0	0.170	0.171	+0.5	0.210	0.212	+0.9
V, "	0.262	0.251	−4	0.2996	0.316	+5	0.3608	0.373	+3
VI, "	0.092	0.0882	−3	0.108	0.105	−2.7	0.124	0.121	−2
VII, "	0.353	0.338	−4	0.379	0.377	−0.5	0.456	0.479	+5
VIII, "	0.280	0.293	+4	0.312	0.325	+2	0.462	0.465	+0.6
IX, cat.	0.735	0.732	−0.4	0.866	0.856	−1	1.220	1.190	−2
X, "	1.15	1.20	+2	1.39	1.452	+4	1.376	1.40	+2
XI, dog.	1.08	1.00	−0.8	1.132	1.162	+2	1.350	1.41	+4
XII, "	0.920	0.89	−3	0.938	0.934	−0.4	1.230	1.20	−2

The standard is prepared at the same time as the unknown and is carried through the same manipulations as regards heating, etc., the only difference being that in place of the urine there is substituted 1 cc. of standard K_2SO_4 equivalent to 0.1 mg. of S.

Determination of Neutral Sulfur.

This fraction is calculated in the usual way by difference between total sulfur and total sulfates.

The methods outlined above have been checked by a series of comparative analyses made by gravimetric methods. Inorganic and total sulfates have been determined by the methods of Folin (2); total sulfur by the procedure of Benedict (3), with the use of Denis' (4) modification of the oxidation mixture em-

ployed in the original method. The results are presented in Table I.

SUMMARY.

Nephelometric methods are described for the rapid quantitative determination of the sulfur compounds in urine. It is believed that these methods may be of use in cases in which the quantity of urine available is limited to an extremely small volume.

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NOTE ON COLORIMETER CORRECTION CURVES.

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Beer's law, the basis of colorimetric determinations, is seldom if ever accurate over wide variations in concentration. The customary method for overcoming this error is the use of a correction curve, prepared experimentally with solutions of known concentration. Such curves have appeared in the literature and are of great value in calling attention to the magnitude of the error, but they evidently must not be taken too literally by the reader.

In an article describing a method (1) (based on that of Folin and Wu (2)) for the determination of sugar in 0.1 cc. of blood, the writer published a correction curve for the error encountered when the concentration of the unknown varies widely from that of the standard. Since that time he has had occasion to doubt the value of such curves when intended for use outside the laboratory in which they were prepared. This method (Folin and Wu) is now so widely used that it would seem worth while to point out the differences between the colorimetric error as found by Rothberg and Evans (3) and by Oser and Karr (4). Their findings, recalculated so that the correction expressed in mg. is plotted against the apparent value as calculated by the ordinary method, are shown in Fig. 1. With them are shown the curves used in this laboratory with standards of 75 and 150 mg. of sugar, also the correction applied by Guillaumin (5) to Folin and Wu's earlier method (6). To avoid confusion the best curve is taken in each case, and no attempt made to indicate the experimental points. As is shown, for example, on the graph, when the observed colorimetric reading is 150 mg. and a standard of 100 mg. is used, the correction to be subtracted is, according to Oser and Karr, 6 mg., to Guillaumin 8 mg., to Rothberg and Evans 13 mg., giving corrected results of 144, 142, and 137 respectively. At other

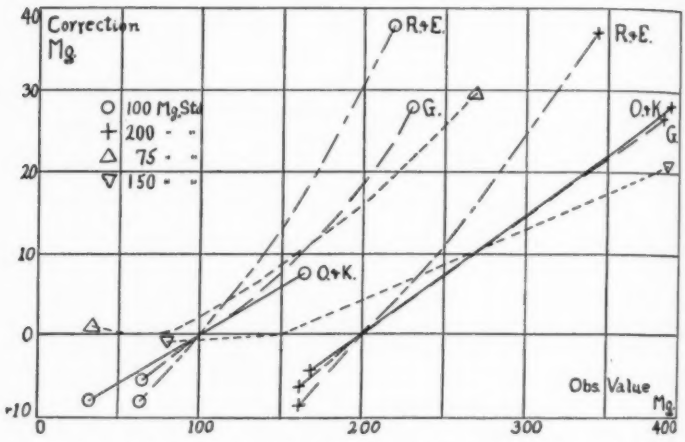

FIG. 1. Colorimeter curves. Folin and Wu sugar determination. Showing the corrections to be *subtracted* from the observed value to obtain the true concentration, as reported by several investigators. The curves found in this laboratory for use with standards of 75 and 150 mg. are included.

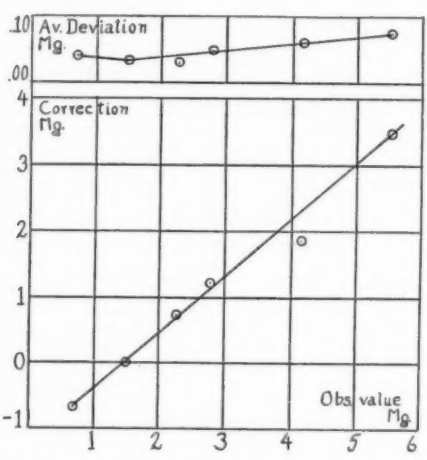

FIG. 2. Colorimeter curve. Creatinine determination. Showing the large correction to be *added* to the observed value to obtain the true concentration.

points the divergence is wider, but the two first named show remarkable agreement when using the standard of 200 mg.

In this laboratory, for clinical purposes, a single standard corresponding to 150 mg. of sugar is used, as it is felt that accurate determinations are of relatively more importance near that concentration, and bloods of greater or less concentration than 150 mg. per 100 cc. are corrected from the graph. The standard of 75 mg. is the one used in micro sugar determinations where the dilution of the blood when precipitated is 20 volumes instead of the usual 10.

Whether the different degree of error is due to personal variation in matching colors that do not exactly match, to differences among colorimeters, or to unconscious differences in technique, would be difficult to decide. It is, however, a simple matter to prepare such a curve, and if the average deviation from the mean at each concentration be calculated, a fair notion of the probable error of any reading may be had.

In the case of blood creatinine determinations by the Folin and Wu method, the use of a curve is even more important than in their sugar method, and Fig. 2 shows the correction curve used in this laboratory for creatinine. The magnitude of this error has not been sufficiently stressed. Here, high creatinine values are corrected by the graph about as far as indicated, and for higher values either the determination is repeated using a diluted filtrate, or, to conserve filtrate, the original colored solution is diluted with a solution containing 1 part of sodium picrate to 2 parts of water until a match is obtained. This was recommended by Folin and Wu (6), and it has been found that all three methods give good agreement. It is not suggested that this graph be used by others without verification.

SUMMARY.

1. Variation among observers of the colorimeter correction curve for sugar (Folin and Wu method) is noted, and the importance of individual determination of these curves stressed.

2. Attention is called to the high colorimetric correction in creatinine determinations, a graph is shown, and other methods of overcoming it are referred to.

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FRACTIONATION OF IRRADIATED CHOLESTEROL.

I. CHEMICAL OBSERVATIONS.*

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Fractionation.

One of us recently reported (1) that cholesterol, as a result of exposure in air to light from the mercury vapor quartz lamp, reacts differently from untreated cholesterol towards a reagent consisting of aniline and concentrated hydrochloric acid. Rosenheim and Webster (2) confirmed this observation. They are of the opinion that this reaction is not specific for vitamin D. How close a connection exists between the chromogenic substance (or substances) and the antirachitic factor remains to be seen. The color reaction is, however, direct chemical evidence of a change in cholesterol following irradiation in air for short periods. Since vitamin D is formed under the same conditions, fractionation of irradiated cholesterol was undertaken with the aid of this color reaction in the hope of obtaining an antirachitically active fraction.

The reagent is made by adding 1 part of concentrated hydrochloric acid to about 15 parts of aniline. Redistilled aniline should be used, as the color of old specimens of aniline obscures the test. The color of the reagent changes on standing. Consequently it should be made up fresh shortly before using. Boiling a small quantity of purified cholesterol with the reagent in a test-tube for about half a minute gives a clear pale yellow solution which remains yellow even on standing more than 24 hours.

* A preliminary report of this investigation was read before the Society for Experimental Biology and Medicine, October 20, 1926 (*Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 48).

When a large quantity of cholesterol is taken for the test, the clear yellow solution solidifies on cooling, but the color remains yellow. After irradiation, however, the cholesterol gives a positive reaction which varies from a yellow with a barely discernible red tinge, through reddish brown to a distinct red, the red color becoming more intense on standing. The intensity of the red color appears to depend on the surface area of the cholesterol exposed to irradiation, the time of irradiation, and on the quantity of irradiated cholesterol tested with a given volume of reagent. This change in reaction towards the aniline-hydrochloric acid reagent is obtained whether the cholesterol is irradiated in acetone solution, or as thin layers formed after evaporation of acetone, methyl alcohol, or petroleum ether solutions of cholesterol.

To rule out the possibility of reaction, under the influence of the irradiation, between the cholesterol and the small amount of organic solvent which might have been adsorbed, thin layers were prepared from aqueous suspension. Irradiation of these films produced a change in behavior towards the aniline-hydrochloric acid reagent similar to that obtained with films prepared from organic solvents. Irradiation in all the experiments reported here was carried out in air at a distance of 18 inches. Two mercury vapor quartz lamps were used interchangeably; one a Burdick and the other a Hanovia lamp, both operating on a circuit of 110 volts. Spectrograms of the light emitted by these lamps, taken with a Hilger quartz spectrograph, showed that the spectra of both lamps extended far into the ultra-violet and that the corresponding lines in each were of approximately equal intensity.

The recrystallization of the cholesterol was performed by dissolving the cholesterol in a small quantity of hot acetone, then adding methyl alcohol, and evaporating off the solvents on a water bath. When most of the cholesterol had crystallized out, the mass was cooled and filtered with suction. The filtrate was concentrated to a smaller volume, cooled, and filtered again. This filtrate was evaporated to dryness and the residue examined. The entire procedure was carried on in the open, exposed to the atmosphere.

Commercial cholesterol, which gave a positive reaction with the aniline reagent, was recrystallized repeatedly to remove im-

purities. After the third recrystallization, the cholesterol gave a negative reaction. The filtrates, however, continued to give a positive reaction until about the sixth recrystallization. This showed that even where the amount of the chromogenic substance in the sterol was too small to be detected with this test, its presence could be shown after concentration in the filtrate. The amount of residue obtained from the filtrate decreased progressively with the first six recrystallizations. After that an approximately constant, small quantity of dry or resinous yellow residue was obtained. The residue from the thirteenth crystallization was about the same in amount as that from the sixth. Since the small amount of residue appeared to become constant, it is possible that it is a substance continuously formed from cholesterol under the conditions of the crystallization.

On recrystallizing some irradiated cholesterol from methyl alcohol, it was noticed that the mother liquor had a faint yellow color. The recrystallized sterol gave a less marked reaction with the aniline-hydrochloric acid reagent, while the alcoholic concentrate gave a more marked reaction than did the unextracted irradiated sterol. When the solvent in the filtrate was removed by evaporation there remained a small quantity of a pale yellow oil.

A sample of cholesterol which had been recrystallized ten times was irradiated for 2 hours and again recrystallized. After evaporation of the solvents from the filtrate, there remained a few drops of a pale yellow oil instead of a minute quantity of dry yellow residue. On cooling to room temperature, the oil became viscous and hardened considerably. A small quantity of this oil gave a marked positive reaction with the aniline-hydrochloric acid reagent. The irradiated sterol after recrystallization reacted negatively. This cholesterol was again irradiated and recrystallized, and again gave a quantity of yellow oil similar in all respects, including amount, to the oil obtained on the previous recrystallization. This irradiation and recrystallization were repeated a number of times on the same sample of cholesterol and each time a considerable quantity of oil was obtained. The same result was obtained with another sample of cholesterol which had first been recrystallized thirteen times. This oil is therefore a

cholesterol derivative, or a mixture of cholesterol derivatives, apparently produced by the irradiation of cholesterol in air. For convenience, it will be referred to as "U. V. oil of cholesterol" until a more suitable name is proposed.

The yield of crude oil was about 10 per cent when the cholesterol was irradiated for 2 hours, and about 5 per cent when irradiated for 1 hour. The crude oil contained an undetermined amount of cholesterol. This residual cholesterol was removed by digitonin precipitation, following essentially the method used by Drummond, Channon, and Coward (3). Since no digitonin was available at the time, the reagent employed was the Hoffman-LaRoche digitalin. This was found to contain about one-tenth of 1 per cent of ether-extractable material. In all cases the addition of the reagent was continued until a test portion of the filtrate from the digitonin precipitate gave a marked turbidity with a solution of cholesterol, thus insuring an excess of digitonin. 1 cc. of the solution, containing 10 mg. of the reagent, was required for every 5 mg. of crude oil before an excess of digitonin could be demonstrated. Since the ratio of digitonin to cholesterol in the precipitate is 3 to 1, it indicated that about 60 per cent of the crude oil was removed by this procedure. The amount of cholesterol-free oil recovered varied from 29 to 57 per cent; the average value for seven different experiments was 42 per cent.

Color Reactions.

With the aniline-hydrochloric acid reagent, the U. V. oil reacted with the same intensity before and after the removal of the residual cholesterol. With Schiff's reagent, freshly purified cholesterol required heat to develop the colors, whereas both the crude and cholesterol-free U. V. oils developed the colors rapidly in the cold. With the Liebermann-Burchard test, 1 mg. of both the crude and cholesterol-free U. V. oils gave decidedly positive results. A flash of colors was often observed, and an intense, bright green color developed rapidly in all cases, although the tint was somewhat different from that given by cholesterol. Cholesterol gave a negative Lifschütz test for oxycholesterol, while a positive test was obtained with irradiated cholesterol and with both the crude and cholesterol-free U. V. oils.

When Salkowski's test was tried on 1 mg. of the cholesterol-free U. V. oil, the rapid play of colors terminating in a violet was obtained in the upper layer, and the marked green fluorescence appeared in the lower layer. With 1 mg. of purified cholesterol no play of colors was obtained; the chloroform layer slowly became yellow, while the acid layer showed only a faint green fluorescence. With larger quantities of cholesterol, the upper layer became yellow and then went over to a red, but no other color appeared; at no time was the green fluorescence in the lower layer pronounced. The green fluorescence was best observed when the test-tube was held in a beam of sunlight and was regarded from the side. A purple color in the upper layer was obtained with cholesterol only after the reaction mixture had remained in the test-tube for some time.

DISCUSSION.

Hess, Weinstock, and Sherman (4) treated irradiated cholesterol with digitonin, and recovered from the filtrate an amorphous yellow residue which was not precipitated by the digitonin. Beumer (5) found that about 8 per cent of the cholesterol, following irradiation for 2 hours, could not be precipitated by digitonin; Hess, Weinstock, and Sherman (6) obtained a 4.5 per cent loss. This latter value is approximately the same as the yield of cholesterol-free U. V. oil which we obtained from cholesterol irradiated for the same length of time. Rosenheim and Webster (2) reported that as much as 10 per cent of air-irradiated cholesterol is not precipitable by digitonin, while with nitrogen-irradiated cholesterol less than 0.1 per cent is not precipitable. When cholesterol, irradiated in nitrogen, was fractionated by crystallization, they found Fraction III to be yellowish. In another experiment with nitrogen-irradiated cholesterol, they removed the cholesterol by digitonin and recovered a yellow resinous solid. Koch, Cahan, and Gustavson (7) separated a small amount of brown resinous material from irradiated cholesterol by extraction with liquid ammonia. Apparently then, some material is formed from cholesterol subsequent to irradiation, whether the irradiation is carried on in air or in nitrogen. As far as the air-irradiated cholesterol is concerned, our experiments show that this substance is an oil which is pale yellow in thin layers and in thicker layers is amber-colored, with an orange tinge.

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There is a lack of agreement in the literature as to just what colors are obtained when Salkowski's test is applied to cholesterol. Salkowski (8) himself stated that the chloroform solution quickly becomes blood-red, then cherry-red, and then purple, the acid layer at the same time exhibiting a strong green fluorescence. We obtained no purple in the upper layer and only a slight amount of green in the acid, even when relatively large amounts, *i.e.* about 100 mg., were taken for the test. With very small amounts of cholesterol Salkowski reported that the colors obtained were "somewhat modified." We found that 1 mg. of cholesterol did not react at all as described above by Salkowski, whereas the play of colors and the brilliant green fluorescence were all obtained with even 0.5 mg. of the U. V. oil.

As stated above, there is a lack of agreement in the literature as to the exact colors obtained with cholesterol in Salkowski's test. A possible explanation for the disagreement is that the cholesterol used by some authors contained impurities, possibly cholesterol derivatives. Specimens of cholesterol may contain enough of an impurity to affect markedly some of its color reactions without any detectable effect on its melting point or other criteria of purity. The purple color mentioned by some authors, and the brilliant green fluorescence mentioned by others, may be due to contamination by cholesterol derivatives such as those present in the U. V. oil. These derivatives may be oxidation products of cholesterol, since the red chloroform layer in the Salkowski test with cholesterol changes to a violet or purple on continued exposure to the air.

In this connection, the work of Lifschütz is of importance. He showed (9) that oxycholesterol interferes in both methods usually used for the determination of cholesterol; *i.e.*, the digitonin precipitation method and the colorimetric method in which the Liebermann-Burchard reaction is employed. He found that with the latter reaction, oxycholesterol, even when entirely free from cholesterol, reacts as does cholesterol itself. He also found that while pure cholesterol is quantitatively precipitated by digitonin, oxycholesterol also is precipitated, although only to about 50 per cent. As a result, neither method, unmodified, can be used for the accurate determination of cholesterol. He suggests a modification of the method, wherein the value for cholesterol is

obtained by difference. In a later communication (10) he gives another method for the quantitative estimation of cholesterol substances in the presence of one another.

More recently Lifschütz (11) demonstrated the presence of oxycholesterol and other cholesterol derivatives in beef blood. About 20 per cent of the unsaponifiable portion of beef blood lipoids he found to consist of cholesterol derivatives which are not precipitable by digitonin. Oxycholesterol was demonstrated in both the digitonin precipitate and in the filtrate. All of these substances, including those not precipitated by digitonin, give the Liebermann-Burchard reaction with great intensity.

We were able to demonstrate the presence of oxycholesterol in both irradiated cholesterol and in the U. V. oil of cholesterol. Whether this oil is composed of a few or of a large number of cholesterol derivatives remains to be seen. The presence of an oxidation product in the U. V. oil of cholesterol gives support to our hypothesis that the changes undergone by cholesterol as a result of irradiation consist of oxidations in which the light acts as catalyst.

By means of a clay catalyst, Bills (12) produced from cholesterol a resinous material which cured experimental rickets. We subjected some of this material, which he kindly sent us, to color tests, and found it to give a positive reaction with the aniline-hydrochloric acid reagent and a negative one for oxycholesterol.

SUMMARY.

1. Irradiation of cholesterol in air results in the formation of a yellow oil, provisionally called "U. V. oil of cholesterol."

2. Cholesterol irradiated in air for 1 hour at 18 inches yields about 5 per cent of crude oil. About 40 per cent of the crude oil is not precipitable by digitonin.

3. The cholesterol-free U. V. oil gives positive reactions with the aniline-hydrochloric acid, Liebermann-Burchard, and Salkowski tests. It also gives the Lifschütz reaction for oxycholesterol.

4. Cholesterol, especially in small quantities, does not give a characteristically positive Salkowski test. This reaction, as described by Salkowski, is more characteristic of the U. V. oil of cholesterol than of cholesterol itself.

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FRACTIONATION OF IRRADIATED CHOLESTEROL.

II. ANTIRACHITIC POTENCY OF THE FRACTIONS.*

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There has been a number of attempts to separate an antirachitically potent fraction from cholesterol irradiated in air. Hess, Weinstock, and Sherman (1), and Rosenheim and Webster (2) tried without success. The latter authors claim to have isolated an active fraction from cholesterol irradiated in nitrogen, but their published data do not seem to warrant such a claim. In one experiment, two fractions obtained by fractional crystallization from nitrogen-irradiated cholesterol gave "partial protection." The number of rats tested is not given. In another experiment on nitrogen-irradiated cholesterol, in testing the potency of the portion not precipitated by digitonin apparently only two rats were used. Besides, conflicting results were obtained, for only one of the two rats showed no rickets. Nitzescu and Popoviciu (3) reported the separation of cholesterol, presumably irradiated in air, into an active and an inactive fraction. Their results were not given in detail so that it is impossible to decide whether their conclusions are warranted. However, their procedure was to feed the Sherman-Pappenheimer Diet 84 for 4 to 12 days, then incorporate the test material in that diet and feed the test material for 3 to 4 weeks. They state that the active fraction *cured* rickets. Inasmuch as the Sherman-Pappenheimer Diet 84 does not produce rickets in 4 to 12 days, the rats could not have had rickets when the test material was first fed and consequently the material could not have cured rickets.

*A preliminary report of this investigation was read before the Society for Experimental Biology and Medicine, October 20, 1926 (*Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 49).

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The preceding paper (4) is a report of the separation of a yellow oil from cholesterol formed as a result of irradiation in air. The antirachitic potency of this "U. V. oil of cholesterol" was tested by means of curative experiments on rachitic rats. At the May, 1926, meeting of the American Pediatric Society, one of us, in discussing a paper by Schlutz and Ziegler, reported (5) that this material cures rickets in rats. During that same discussion, Julius H. Hess (6) stated that Koch had obtained a potent extract from irradiated cholesterol by means of liquid ammonia. Previously, Koch, Cahan, and Gustavson (7) obtained a brown, resinous material from irradiated cholesterol by this method, but did not report as to its antirachitic potency.

Very recently, Hess, Weinstock, and Sherman (8) also reported the separation of irradiated cholesterol into an active and an inactive fraction. The separation of the cholesterol digitonide from the soluble fraction was carried out in an atmosphere of nitrogen.

EXPERIMENTAL.

Commercial cholesterol was repeatedly recrystallized from acetone and methyl alcohol until the mother liquor gave a negative reaction with the aniline-hydrochloric acid reagent. A hot acetone solution of the cholesterol was poured on shallow pans and the solvent allowed to evaporate. The thin films thus obtained were irradiated in air for 1 hour at 18 inches. The crude U. V. oil was extracted from the irradiated cholesterol, and the cholesterol-free U. V. oil was prepared from the crude oil as described in the preceding communication (4). The substances tested for antirachitic potency were prepared from freshly irradiated cholesterol and then immediately incorporated in the diet and fed to the rats.

Rats weighing about 25 to 45 gm. were put on the Steenbock Diet 2965 when they were about 25 to 35 days old. They were kept on this diet for 17 to 31 days. Before feeding the test material a few rats were autopsied to determine whether the series had developed marked rickets. These served as initial controls. When the initial controls showed marked rickets, the remaining rats of the series were divided into groups, weighed, and then fed the test diets for about 11 days. One group was continued on the

TABLE I.

Date autopsied.	Rat No.	Normal diet.	Steenbock Diet 2965.	Test.	Test period.	Histological findings.
1920		*days*	*days*		*days*	
May 19	74	28	17	0.01 per cent crude U. V. oil.	5	No healing.
	75	28	17	0.01 " " " " "	5	Moderate healing.
	83	28	17	0.01 " " " " "	5	No healing.
	84	28	17	0.01 " " " " "	5	" "
May 19	76	28	17	Steenbock.	5	No healing.
	77	28	17	"	5	" "
May 27	78	28	17	2 per cent crude U. V. oil plus cottonseed oil.	5	Advanced healing.
	79	28	17	2 " " " " " " "	5	" "
	82	28	17	2 " " " " " " "	5	" "
	85	28	17	2 " " " " " " "	5	" "
May 27	80	28	17	Steenbock plus cottonseed oil.	5	No healing.
	81	28	17	" " "	5	" "

TABLE II.

Date autopsied.	Rat No.	Normal diet.	Steenbock Diet 2965.	Test.	Test period.	Weight.		Histological findings
						Initial.	Final	
1926		*days*	*days*		*days*	*gm.*	*gm.*	
June 17	137	28	28		*	50		Marked rickets.
	138	28	28			55		" "
	139	28	28			52		" "
	140	28	28			50		" "
June 28	153	28	29	0.01 per cent irradiated cholesterol.	9	75	85	Beginning healing.
	154	28	29	0.01 " " " "	9	75	81	" "
	155	28	29	0.01 " " " "	9	67	76	No healing.
	156	28	29	0.01 " " " "	9	72	78	" "
	158	28	29	0.01 " " " "	9	71	81	Beginning healing.
July 1	147	28	29	0.01 per cent crude U. V. oil.	12	80	89	No healing.
	148	28	29	0.01 " " " " " "	12	70	78	" "
	149	28	29	0.01 " " " " " "	12	73	81	" "
	150	28	29	0.01 " " " " " "	12	62	69	" "
	151	28	29	0.01 " " " " " "	12	59	58	" "
	152	28	29	0.01 " " " " " "	12	65	71	" "
July 2	159	28	29	0.01 per cent cholesterol-free U. V. oil.	13	70	75	Beginning healing.
	160	28	29	0.01 " " " " " " "	13	63	65	No healing.
	162	28	29	0.01 " " " " " " "	13	65	69	" "
	164	28	29	0.01 " " " " " " "	13	74	89	" "

July 1	172	28	30	0.2 per cent recrystallized irradiated cholesterol.	12	74	82	No healing.
	173	28	30	" "	12	72	77	" "
	174	28	30	" "	12	75	81	" "
	175	28	30	" "	12	63	67	" "
	176	28	30	" "	12	72	80	" "
July 1	141	28	29	0.1 per cent crude U. V. oil.	12	89	106	Moderate healing.
	142	28	29	0.1 " " " " " "	12	63	67	No healing.
	143	28	29	0.1 " " " " " "	12	80	96	" "
	144	28	29	0.1 " " " " " "	12	70	76	" "
	145	28	29	0.1 " " " " " "	12	65	69	" "
	146	28	29	0.1 " " " " " "	12	80	88	Advanced healing.
July 1	166	28	30	0.1 per cent cholesterol-free U. V. oil.	12	67	80	Advanced healing.
	168	28	30	0.1 " " " " " "	12	58	63	Moderate "
	170	28	30	0.1 " " " " " "	12	73	83	" "
July 2	165	28	30	0.1 " " " " " "	13	55	52	Beginning "
	167	28	30	0.1 " " " " " "	13	73	80	" "
	169	28	30	0.1 " " " " " "	13	57	57	" "
July 2	177	28	29	Steenbock.	14	63	63	No healing.
	178	28	29	"	14	63	66	" "
	179	28	29	"	14	69	78	" "
	180	28	29	"	14	78	85	" "
	181	28	29	"	14	70	81	" "

* Initial controls.

rickets diet throughout the test period and served as a terminal control. At the end of the experiment all the animals were autopsied and examined macroscopically. Sections of the proximal ends of the tibiæ were stained with a 1 per cent silver nitrate solution and examined with the binocular microscope.

By "beginning healing" is meant a scattered deposition of calcium salts at the tips of the cartilaginous processes as shown by the silver nitrate stain; by "moderate healing," a definite line of newly deposited salts in the provisional zone of calcification, extending across the width of the bone section—the "line-test" of McCollum and his coworkers; by "advanced healing," the calcification of the provisional zone and about two-thirds of the metaphysis; while by "complete healing" is meant deposition of calcium throughout the metaphysis and zone of provisional calcification,—the metaphysis nearly disappearing, the zone of cartilage assuming its normal width, and the bone approaching its normal architecture.

Some rats became paralyzed and lost considerable weight during the test period, and consequently were discarded. Most of these animals were on diets which contained no curative material. Subsequent experiments have shown us that a very severe rickets is produced with our technique in 17 to 20 days. Prolongation of the period on the rickets-producing diet sometimes resulted in paralysis, and a few days later, in death. This paralysis may be due to the severity of the rickets. To avoid these losses it may be advisable to start the test period earlier—after about 20 days on the Steenbock diet.

In the first experiment, an alcoholic solution of the crude U. V. oil was mixed with the diet and the ethyl alcohol removed by evaporation. Table I shows that after 5 days on a 0.01 per cent level of crude U. V. oil there was healing in only one of the four animals. This suggested that either the amount of crude U. V. oil was below the minimum for healing, or that exposure of the oil resulted in loss of potency. For this reason, the second group was fed crude U. V. oil at a 2 per cent level, protected by cottonseed oil, amounting to 3 per cent of the diet. This was fed for 5 days after 7 days on a 0.005 per cent level of crude U. V. oil. In this group advanced healing was obtained in all four rats (Table I).

In the second experiment, one group was killed at the beginning

of the test as an initial control group, and another at the end of the experiment, as a terminal control group. All the initial controls showed marked rickets; all the terminal controls showed marked rickets with no evidence of healing.

From Table II it is seen that irradiated cholesterol at 0.01 per cent level produced beginning healing in 9 days in only three out of five rats. Crude U. V. oil had no curative effect in 12 days at 0.01 per cent level. Cholesterol-free U. V. oil at 0.01 per cent level produced beginning healing in one rat out of four. At 0.1 per cent level, crude U. V. oil produced healing in two rats out of six, while the cholesterol-free U. V. oil produced healing in all six animals. Some of the irradiated cholesterol was recrystallized from acetone and methyl alcohol. The recrystallized irradiated cholesterol, which reacted negatively with the aniline-hydrochloric acid reagent, was fed at a 0.2 per cent level and induced no healing.

The efficacy of the mercury vapor quartz lamp used was tested by animal experiments as well as by the spectrograms mentioned in the preceding communication. Four animals were irradiated directly with increasing dosage over a period of 15 days. This group showed almost complete healing of the rickets (Table III).

In this experiment, four rats fed with irradiated cholesterol at 0.3 per cent level showed good healing. With 0.1 per cent level of crude U. V. oil protected by cottonseed oil, there was healing in two cases out of four; with 0.3 per cent, there was good healing in all five cases. With 0.3 per cent cholesterol-free U. V. oil protected by cottonseed oil, all five rats showed good healing.

To make sure that the cottonseed oil did not induce healing, additional tests were performed (Table IV). As expected, no healing was obtained with cottonseed oil.

In the last experiment (Table V) the effect of the reagent on the curing of rickets was tested. Since digitalin was used as the source of digitonin, and since about one-tenth of 1 per cent of the digitalin was ether-extractable, the possible curative effect of this material had to be ruled out. Three rats were fed the rickets diet to which had been added twice the quantity of ether extract of digitalin as would have found its way into the diet in the preparation of a 0.3 per cent level of cholesterol-free U. V. oil diet. None

TABLE III.

Date autopsied.	Rat No.	Normal diet.	Steenbock Diet 2965.	Test.	Test period.	Weight.		Histological findings.
						Initial.	Final.	
1926		days	days		days	gm.	gm.	
June 29	182	25	22		*	50		Marked rickets.
	183	25	22			53		" "
July 22	203	25	29	Direct irradiation.	15	50	57	Complete healing.
	204	25	29	" "	15	47	46	" "
	205	25	29	" "	15	43	51	" "
	206	25	29	" "	15	44	52	Advanced "
July 12	184	25	26	0.3 per cent irradiated cholesterol.	9	55	59	Moderate healing.
	185	25	26	0.3 " " " "	9	61	60	" "
	187	25	26	0.3 " " " "	9	63	69	Advanced "
	188	25	26	0.3 " " " "	9	41	44	Moderate "
July 19	207	25	31	0.1 per cent crude U. V. oil plus cottonseed oil.	11	67	65	Moderate healing.
	208	25	31	" "	11	42	47	No healing.
	209	25	31	" "	11	45	52	Beginning healing.
	210	25	31	" "	11	46	47	No healing.
July 19	198	25	31	0.3 per cent crude U. V. oil plus cottonseed oil.	11	62	64	Advanced healing.
	199	25	31	" "	11	50	51	Moderate "
	200	25	31	" "	11	46	51	Advanced "
	201	25	31	" "	11	63	67	" "
	202	25	31	" "	11	62	62	Moderate "

	202	25	31	"	"	11	63	67	" "
						11	62	62	Moderate "
July 20	193	25	30	0.3 per cent cholesterol-free U. V. oil plus cottonseed oil.		13	45	47	Advanced healing.
	194	25	30	"	"	13	54	57	Moderate "
	195	25	30	"	"	13	53	56	Advanced "
	196	25	30	"	"	13	66	70	" "
	197	25	30	"	"	13	45	48	Moderate "
July 23	258	25	29	Steenbock.		13	28†	50	No healing.
	259	25	29	"		13	32	59	" "
	260	25	29	"		13	25	48	" "
	261	25	29	"		13	25	39	" "

* Initial controls.

† Weight at the beginning of the 42 day period on Steenbock diet.

TABLE IV.

Date autopsied.	Rat No.	Normal diet.	Steenbock Diet 2965.	Test.	Test period.	Weight.		Histological findings.
						Initial.	Final.	
1926		days	days		days	gm.	gm.	
July 22	254	25	23	Steenbock plus cottonseed oil.	*	88		Marked rickets.
Aug. 2	252	25	23	" " " "	11	52	55	No healing.
	253	25	23	" " " "	11	72	76	" "
	255	25	23	" " " "	11	45	50	" "
	256	25	23	" " " "	11	49	49	" "
	257	25	23	" " " "	11	72	74	" "
Sept. 11	288	37	23	Steenbock plus cottonseed oil.	13	48†	77	No healing.
	289	37	23	" " " "	13	42	60	" "
	290	37	23	" " " "	13	45	66	" "
	294	37	23	" " " "	13	37	54	" "
	295	37	23	" " " "	13	41	65	" "
	296	37	23	" " " "	13	37	65	" "
	293	37	23	" " " "	13	40	52	" "

* Initial control.

† Weight at beginning of 36 day period on Steenbock diet.

TABLE V.

Date autopsied.	Rat No.	Normal diet.	Steenbock Diet 2965.	Test.	Test period.	Weight.		Histological findings.
						Initial.	Final.	
1926		*days*	*days*		*days*	*gm.*	*gm.*	
Sept. 26	324	31	25	Ether extract of digitalin.	10	58	62	No healing.
	325	31	25	" " " "	10	51	55	" "
	326	31	25	" " " "	10	57	66	" "
Sept. 26	328	31	23	0.3 per cent cholesterol-free U. V. oil plus cottonseed oil (stored 2 months).	12	45	53	Moderate healing.
	329	31	23	" "	12	46	54	" "
Sept. 26	330	31	23	Steenbock.	12	41	43	No healing.
	331	31	23	"	12	36	45	" "

of the three showed any healing. Two rats were fed a diet containing 0.3 per cent cholesterol-free U. V. oil. This mixture had been made up 2 months previously and had been found potent. A quantity just sufficient for a test with two rats had remained from the first test. After standing for 2 months, it was still potent, as evidenced by the healing induced in both rats. The two control rats showed marked rickets, with no evidence of healing.

DISCUSSION.

Steenbock and Black (9) reported that irradiated cholesterol cures rickets even when fed at 0.01 per cent level. At this level we obtained healing in only three out of five rats, and the healing was only in the initial stage. This difference between our results and theirs may be due to slight differences in technique. At any rate, 0.01 per cent level is in the neighborhood of the minimum dose of irradiated cholesterol necessary to induce healing in 9 days. In accordance with the results reported by Hess, Weinstock, and Sherman (1), and subsequently confirmed by others, we found that irradiated cholesterol lost its antirachitic potency following recrystallization. Even at 0.2 per cent level this recrystallized cholesterol showed no curative powers. Crude U. V. oil of cholesterol when fed at 0.1 per cent level produced healing in only two cases out of four, even when protected by cottonseed oil, whereas at 0.1 per cent level, the cholesterol-free U. V. oil induced healing in six cases out of six. Since the rats consumed on the average about 5 gm. of diet per day, 0.1 per cent is equivalent to 5 mg. of cholesterol-free U. V. oil per rat per day. Of course, if the U. V. oil retained all the potency of the irradiated cholesterol from which it was prepared, it should cure rickets when given in much smaller doses. However, the extraction was carried out in the open, and since the antirachitic factor seems to be destroyed on exposure to the air, it is surprising that even as small a dose as 5 mg. per rat per day is sufficient to induce healing. With 0.3 per cent of the U. V. oil in the diet, marked healing was obtained with both the crude and cholesterol-free oils.

SUMMARY.

1. Crude U. V. oil of cholesterol cures experimental rickets in rats.

2. Cholesterol-free U. V. oil of cholesterol cures experimental rickets when fed at 0.1 per cent level.

3. The antirachitic effect of the U. V. oil is not due to material extracted by ether from the reagent employed for the removal of the cholesterol.

4. Cholesterol-free U. V. oil of cholesterol, protected by cottonseed oil and incorporated in the diet, retains its antirachitic potency after 2 months storage.

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XUM

GASOMETRIC MICRO-KJELDAHL DETERMINATION OF NITROGEN.

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The ease and accuracy with which the manometric blood gas apparatus (4) makes possible the measurement of small amounts of gas evolved from solutions has led us to extend the use of the apparatus to determinations of substances other than the blood gases. The apparatus can be used in any determination in which the final product is a gas, or will enter into a quantitative reaction producing a gas. Thereby the measurement is based, as in gravimetric analysis, on direct observation of the amount of substance obtained, independent of comparison with standard solutions by titration, colorimeter, or otherwise. This advantage of directness and freedom from dependence upon the accuracy of standards is combined with the rapidity and ease of a final measurement by volume.[1]

[1] Technique with the manometric gas apparatus has been developed for exact urea determinations by measurement of the CO_2 of the ammonium carbonate formed with urease, and for approximate urea determinations for the hypobromite reaction; for calcium determination by precipitation of the calcium as oxalate, resolution in dilute sulfuric acid, and determination of the CO_2 evolved by the practically instantaneous oxidation of oxalic acid with permanganate in the gas apparatus; for amino nitrogen by the nitrous acid reaction; for sugar by permitting it to reduce potassium ferricyanide, and measuring the excess ferricyanide by the N_2 evolved from the reaction with hydrazine. These methods will be reported shortly. We have also attempted to determine phosphoric acid by estimating with hypobromite the ammonia of the magnesium ammonium phosphate precipitate, and, following a friendly suggestion by A. T. Shohl, the ammonia of the yellow ammonium phosphomolybdate precipitate obtained according to Lorenz. The ammonia contents of both precipitates under the conditions thus far tried have, however, been sufficiently variable to cause errors up to as much as $\pm$ 7 per cent.

XUM

In this paper a method for micro-Kjeldahl determination of nitrogen is reported which has proven satisfactory for both experimental work and hospital routine. The ammonia in the Kjeldahl digest is estimated gasometrically by treatment with hypobromite in the manometric apparatus of Van Slyke and Neill. The pressure of 1 mg. of nitrogen measured at 2 cc. volume in the apparatus is about 300 mm., so that accurate readings are easy: duplicate results usually check to within 1 per cent.

The gasometric determination of ammonia by measurement of the nitrogen gas evolved by the reaction with hypobromite,

$$2NH_3 + 3NaBrO = 3H_2O + 3NaBr + N_2,$$

has been a standard procedure of recognized accuracy for at least 50 years (1). The amount of nitrogen gas evolved falls somewhat short of that calculated by the above equation, but the percentage of the theoretical yield is so constant under defined conditions of hypobromite and alkali concentration that with the introduction of an empirical correction factor Classen states the error may without special precautions be kept within 0.5 per cent of the ammonia determined.

Stehle (2) has used the hypobromite reaction in the original constant pressure blood gas apparatus of the writer to determine the ammonia nitrogen obtained in micro-Kjeldahls. Stehle found (3) that copper used as a catalyst for the sulfuric acid digestion reacted with the hypobromite to cause evolution of appreciable amounts of oxygen gas, which had to be absorbed with pyrogallol before the nitrogen volume could be observed. When this precaution was observed, theoretical results were obtained with sodium sulfanilate, uric acid, and urea, and results consistent with the macro-Kjeldahl for casein and milk. However, the absorption of oxygen was a troublesome additional step in the procedure and Stehle finally (3) abandoned the use of copper catalyzer, and carried out the digestion with only sulfuric acid and potassium sulfate. He found that this procedure gave satisfactory results for non-protein nitrogen determination in blood filtrates.

Our experience confirms Stehle's, that with the blood gas apparatus ammonia determinations by the hypobromite method may be carried out with accuracy. Without catalysts or oxidizing agents to assist in the Kjeldahl digestion, however, the use of the method

for micro-Kjeldahls would be very limited. Low results are obtained with proteins and other substances which offer resistance to digestion. The problem of performing a simple gasometric nitrogen determination therefore resolved itself into finding a satisfactory general catalyzer for the digestion which would not prevent the reaction of the ammonia with hypobromite, nor cause formation of gases other than the nitrogen evolved from the ammonia. Various catalysts and oxidizers previously employed for Kjeldahl determinations were tested. Mercury was found to form a combination with ammonia which resists the action of hypobromite. Perchloric acid if used in excess destroyed part of the ammonia during the digestion. Hydrogen peroxide gave inconstant results. However, when potassium persulfate in great excess as used by Wong (5), was tried, satisfactory results were obtained with regularity. The digestion even of protein material is completed in a few minutes, and with less resistant material such as urine or blood filtrate oxidation is completed so quickly that the dark color of carbon does not even appear. Furthermore, persulfate does not destroy any ammonia. Unnecessary prolongation of the digestion consequently does not injure the results, provided, of course, the overheating is not carried to the extreme of volatilizing ammonium sulfate.

About the only point that requires especial consideration when persulfate is used is the water content of the digest at the time the persulfate is added. If the material to be analyzed is dissolved in several cc. of water per cc. of acid used, and sulfuric acid and persulfate are both added before the water is boiled off, all of the persulfate will decompose during the subsequent boiling down, and before the solution has become concentrated enough for digestion to occur. Results will accordingly be low because of lack of oxidizing agent during the actual digestion. This was pointed out by Wong (5). On the other hand, if the sulfuric acid solution is boiled down till SO_3 fumes come off, and then dry persulfate is added, the latter will not be completely decomposed during the digestion, and will evolve appreciable amounts of oxygen when treated with hypobromite. The mixture of ordinary concentrated sulfuric acid and syrupy phosphoric acid, 3:1, which we finally came to use, appears to have enough moisture in it to act with the persulfate on a dry substance; and 1 cc. of water is not too much.

The procedure adopted for samples dissolved in more than 1 cc. of water is accordingly to boil down with 1 cc. of the acid mixture till fumes began to come off, and to add 1 gm. of persulfate, with 1 cc. of water, to the cooled residue, before finishing the digestion.

The use of phosphoric acid in the digest is not necessary but it accelerates the digestion. 0.1 cc. of blood serum digested with 1 cc. of sulfuric acid and 1 gm. of persulfate requires about 15 minutes to clear. If a mixture of 1 part phosphoric to 3 parts of sulfuric acid is used, instead of sulfuric alone, 5 minutes suffice. A larger proportion of phosphoric acid is likely to attack the glass tubes.

The hypobromite solution used was an object of considerable experiment. Various concentrations of bromine in various concentrations of alkali have been used in the past. All of them form oxygen while standing. Stehle's solution, containing per 100 cc. 2.5 gm. of Br, 2.5 gm. of KBr, and 5.3 gm. of NaOH, was found to form oxygen several times as fast as solutions of bromine in more concentrated NaOH without bromide. The solution finally adopted, 1 cc. of bromine to 50 cc. of 40 per cent sodium hydroxide, generates oxygen so slowly that the amount formed in an hour in 1 cc. of the solution (the amount used for an analysis) exerts at 2 cc. volume only about 0.3 mm. pressure. The presence of 40 gm. of NaOH per 100 cc. reduces the solubility of air in the solution to so nearly zero that it is not necessary to extract the air before the solution is used, the slight trace of air present being included in the blank for the reagents. The amount of bromine present in the 1 cc. of this solution is sufficient to decompose in 1 minute the maximum amount of ammonia for which the method is designed, and it is not enough to cause trouble by the rate of its attack on the mercury in the gas apparatus.

Description of Method.

Reagents.

Sulfuric-Phosphoric Acid Mixture.—3 volumes of concentrated sulfuric acid are mixed with 1 volume of syrupy phosphoric acid (specific gravity 1.7).

Potassium persulfate containing less than 0.01 mg. of ammonia nitrogen per gm.[2]

40 Per Cent Sodium Hydroxide.—40 gm. of NaOH per 100 cc. of solution, ammonia-free.

Sodium Hypobromite Solution.—To 50 cc. of the 40 per cent NaOH in a 250 cc. flask add under a hood, 1 cc. of bromine, and stir until the bromine is completely dissolved. Gaseous oxygen forms very slowly in this solution. In order to prevent some slight accumulation, however, the hypobromite is aerated by whirling it about the walls of the flask for a few seconds each time before a portion is used for analysis. *The hypobromite solution is made fresh on the same half day in which it is used.*

Procedure.

Digestion.—In a 200 × 25 mm. test-tube of Pyrex glass or silica, place the sample to be analyzed, containing preferably 0.3 to 1.5 mg. of nitrogen, 1 cc. of the sulfuric-phosphoric acid mixture, and a glass bead to prevent bumping. If the volume of water added with the sample is 1 cc. or less, 1 gm. of potassium persulfate is added at once. If a larger volume of water is present, however, it is first boiled down till white fumes appear, the mixture is cooled, and 1 gm. of persulfate is then added, together with 1 cc. of water. Heating is continued until the mixture has become entirely clear, or after white fumes appear, in the case of substances which oxidize so readily that the digest is clear throughout the period of heating. A micro burner is used, and during the final

[2] Ordinary commercial $K_2S_2O_8$ contains much ammonia. It may be purified as follows. The persulfate is dissolved in 10-fold its weight of water previously warmed to 60–70°. The solution is made strongly alkaline to litmus by addition of 40 per cent NaOH, is transferred to a vacuum distilling apparatus, and distilled under a pressure of less than 40 mm. When about one-third of the solution has been distilled off, it is replaced by an equal volume of water at 60–70°, more alkali is added if necessary to restore alkalinity to the solution, and the distillation is continued until the distillate tested by Nessler's reagent is ammonia-free. The addition of warm water and alkali may have to be repeated again before all the ammonia is removed. When ammonia has been completely removed, the persulfate solution is poured into a beaker and let stand overnight, preferably in an ice box, to crystallize. The crystals are washed with cold water on a Buchner funnel, and dried over sulfuric acid in a vacuum desiccator. Persulfate prepared this way can be obtained from Merck and Co.

heating somewhat less heat is used than in the ordinary micro-Kjeldahl-Gunning digestion. The tip of the burner should just touch the bottom of the test-tube. An unnecessary intensity or prolongation of the heating may cause bumping, or corrosion of the test-tube by the phosphoric acid in the digestion mixture.

Occasionally a substance unusually difficult to digest (*e.g.* a lipoid) may fail to clear in 15 minutes. In such a case the digest is cooled somewhat, and 0.2 to 0.3 gm. more of potassium persulfate with 2 or 3 drops of water is added.

Neutralization of Digest and Transfer to Gas Apparatus.—The digest is cooled, 3 cc. of water are poured upon it, and a drop of 1 per cent alizarin sulfonate is added. From a pipette 40 per cent sodium hydroxide is then added, a drop at a time, with occasional cooling of the tube in a cold water stream. The heat of the reaction causes solution of the solidified melt in the bottom of the tube. Addition of alkali is continued until the mixture becomes alkaline to the alizarin. 10 per cent sulfuric acid is then dropped in until the color just changes back to acid, in order to prevent loss of ammonia during subsequent deaeration.

The solution, cooled to room temperature, is poured into the cup of the 50 cc. Van Slyke-Neill blood gas apparatus, and the volume is noted. The solution is then drawn down into the chamber. Into a graduated pipette is drawn enough water to make the total volume of the solution up to 10 or 11 cc., and this water is used in three portions to rinse into the gas apparatus the drops of solution left in the test-tube.

Gasometric Determination of Ammonia Nitrogen.—The cock of the gas chamber is sealed with a drop of mercury, the chamber is evacuated, and is shaken 2 minutes to extract the air from the solution. The extracted air bubble is ejected.[3] The extraction is

[3] The most satisfactory technique for ejecting the gas bubble without any solution is to bring the pressure in the chamber to atmospheric by connecting the chamber with the leveling bulb and holding the latter at such a height that the mercury surfaces in chamber and bulb are on the same level. The cock connecting with the leveling bulb is then closed, and the cock at the top of the chamber is opened wide. The contents of the chamber remain quiet as the pressure was already atmospheric. Now with the leveling bulb resting in the ring above the chamber level, the cock connecting bulb and chamber is slowly opened. The fluid in the chamber rises slowly until by closing the cock the rise is stopped when the water has filled the capillary at the top of the chamber.

repeated, yielding usually a very small second air-bubble, which also is ejected without permitting more than a drop of the solution to follow it up into the cup of the apparatus.

The hypobromite solution is whirled for a few seconds about the walls of its flask to permit escape of any oxygen gas that has formed in it, and 1.5 cc. are transferred to the cup of the gas apparatus. 1 cc. is run down into the chamber. The remaining 0.5 cc. is removed from the cup, and the cock is sealed with mercury. The chamber is then evacuated and shaken 2 or 3 minutes.

The gas volume is reduced to 2 cc. and the reading, p_1 mm., on the manometer is noted, together with the temperature.

The gas is then ejected from the chamber,[3] the meniscus of the solution is lowered again to the 2 cc. mark, and p_2 is read on the manometer.

When sufficient material for duplicate analyses is available, one may take an amount of material containing 1 to 4 mg. of nitrogen, with 2 cc. of sulfuric-phosphoric acid mixture and 2 gm. of persulfate. The digestion is carried out in a tube marked at 25 cc. capacity. After neutralization the digest is diluted up to the mark, and 10 cc. aliquot portions are taken for gasometric nitrogen determinations.

Calculation.

$$\text{Mg. N} = (p_1 - p_2 - c) \times f$$

where f is the factor indicating the mg. of N corresponding to 1 mm. of pressure at the temperature noted. The factor is found in Table I.

$p_1 - p_2 - c$ = pressure of nitrogen gas from the substance analyzed.

c is the value of $p_1 - p_2$ obtained in a blank analysis of the reagents, which are digested with 1 cc. of water and otherwise treated as in the analysis described. With good reagents the blank, c, does not exceed 6 mm.

Under the conditions given, the hypobromite yields 96 per cent of the theoretical amount of nitrogen gas. Consequently the factors in Table I give the weight of N_2 measured in the apparatus multiplied by 1.04.

In order to obtain the factors in Table I, the factors in Table III of Van Slyke and Neill marked "Factors for O_2, CO, and N_2, Sample = 1 cc., S = 3.5 cc., a = 2.0 cc., i = 1.00" were multiplied by $\frac{28.08}{1000} \times 1.04 = 0.0292$.

TABLE I.

*Factors by Which Millimeters of Pressure Measured at 2 Cc. Volume Are Multiplied to Obtain Mg. of Nitrogen.**

Temperature.	Factor.
°C.	
15	0.003250
16	41
17	27
18	15
19	01
20	3192
21	80
22	68
23	56
24	45
25	36
26	24
27	16
28	3004
29	92
30	80
31	71
32	60
33	50
34	40

* These factors include the empirical correction factor of 1.04, for the reaction with hypobromite.

Applications to Blood and Urine Analysis.

For determination of the total nitrogen of serum it is convenient to dilute 10-fold and digest 1 cc. of the solution, equivalent to 0.1 cc. of serum.

For albumin nitrogen in the filtrate obtained by Howe's method, in which 0.5 cc. of plasma is added to 15 cc. of 22 per cent Na_2SO_4,

5 cc. of the filtrate are boiled down with 1 cc. of the sulfuric-phosphoric acid mixture till fumes appear. The mixture is cooled. 1 cc. of water and 1 gm. of potassium persulfate are added, and the digestion is continued as usual. Because of the sodium sulfate present it is well to add 5 cc. of water before neutralizing, to avoid crystallization of the salt in the tube. The final volume of fluid transferred to the gas apparatus is therefore 13 to 14 instead of 10 to 11 cc., but the results are not affected.

Non-protein nitrogen of blood is done on 10 cc. of trichloroacetic acid or Folin-Wu filtrate, which is boiled down in the tube with 1 cc. of sulfuric-phosphoric acid mixture before adding the persulfate plus a few drops of water.

Urines if of ordinary concentration are diluted 5-fold, if concentrated, 10-fold, and 1 cc. samples of the solution are digested.

EXPERIMENTAL.

Analyses of Pure Substances.

Substances readily soluble in water or dilute alcohol were weighed out in amounts of 100 or 200 mg. and diluted to 20 cc. 1 cc. portions, containing 5 or 10 mg. of substance, were then analyzed as above described; *viz.*, 1 cc. of 3:1 sulfuric-phosphoric acid mixture and 1 gm. of potassium persulfate were added, and the mixture was heated until it had cleared. The ammonia nitrogen was then determined with hypobromite.

Substances not readily soluble in water were weighed out in portions of 20 to 30 mg. into Pyrex test-tubes. The substance was washed down to the bottom of the tube with as little water as possible, 2 cc. of the 3:1 sulfuric-phosphoric acid mixture were added, and the mixture was heated till fumes began to come off. It was then cooled, and 2 gm. of potassium persulfate with 1 cc. of water were added. The mixture was heated 7 to 9 minutes after it had cleared. (It was later found that this heating after clarification is unnecessary.)

4 or 5 cc. of water were poured over the digest, which was then neutralized as usual with 40 per cent NaOH. It was finally diluted up to 25 cc. volume and 10 cc. portions were transferred to the gas apparatus for hypobromite treatment. The results are given in Table II.[4]

[4] The preparations of the last four substances in the table were kindly furnished by Dr. W. A. Jacobs.

TABLE II.

Analysis of Known Substances.

Substances.	Preparation of solution for analysis.	Weight of substance analyzed.	Pressure of N_2 at 2 cc. volume.	Temperature.	Nitrogen found (with factors from Table I).	Nitrogen found.	Nitrogen calculated.	Time required for digest to clear after beginning to char.
		mg.	*mm.*	*°C.*	*mg.*	*per cent*	*per cent*	*min.*
Ammonium sulfate, $(NH_4)_2SO_4$.	0.9175 gm. to 200 cc. 2 cc. digested and diluted to 25 cc. 10 cc. for analysis.	3.670	252.4	24.0	0.794	21.63	21.22	Did not char.
			253.0	24.0	0.798	21.73		
		3.670	252.8	24.2	0.795	21.67		
			252.9	24.5	0.794	21.63		
Urea, CH_4ON_2.	150 mg. to 100 cc. 2 cc. for digestion and analysis.	3.00	442.6	24.0	1.392	46.4	46.7	Did not char.
			441.3	24.0	1.387	46.2		" " "
Glycocoll, $C_2H_5O_2N$.	100 mg. to 20 cc. 1 cc. for digestion and analysis.	5.00	299.5	22.5	0.948	18.96	18.70	Did not char.
			303.3	23.0	0.958	19.16		" " "
			300.0	23.8	0.946	18.92		
Leucine, $C_6H_{13}O_2N$.	100 mg. to 20 cc. 1 cc. for digestion and analysis.	5.00	164.4	20.5	0.526	10.52	10.70	1
			161.8	20.5	0.516	10.32		
			164.2	21.0	0.523	10.46		
			164.3	21.0	0.523	10.46		

Glutamic acid hydrochloride, $C_5H_{10}O_4N$ HCl.	200 mg. to 20 cc. 1 cc. for digestion and analysis.	10.00	242.1 244.8 246.6	21.3 21.0 21.5	0.766 0.778 0.782	7.66 7.78 7.82	7.60	7–8
Acetanilide, C_8H_9ON.	200 mg. in 50 per cent alcohol to 20 cc. 1 cc. for digestion and analysis.	10.00	331.6 332.0	24.0 24.0	1.058 1.059	10.58 10.59	10.38	2
Hydroxy-6-ethoxy-acetanilide, $C_{10}H_{13}O_3N$.	27.5 and 25.4 mg. digested and diluted to 25 cc. 10 cc. for analysis.	11.00 10.17	253.6 234.6	25.6 25.8	0.793 0.734	7.20 7.22	7.19	Not recorded.
Dimethoxychlorodiethylbenzylamide, $C_{11}H_9O_2N_3$.	24.5 and 26.4 mg. digested and diluted to 25 cc. 10 cc. for analysis.	9.80 10.56	177.2 191.1	19.5 20.0	0.549 0.609	5.60 5.76	5.77	Not recorded.
m-Uramino-benzamide, $C_8H_9O_2N_3$.	23.2 and 21.6 mg. digested and diluted to 25 cc. 5 cc. for analysis.	4.64 4.32	337.6 316.0	23.3 24.0	1.064 0.984	22.92 23.01	22.95	Not recorded.

Analyses of Urines.

Urines were diluted 5-, or 10-, or 15-fold, according to their apparent concentration, and 1 cc. samples, equivalent to 0.2, 0.1, or 0.0667 cc. of urine, were analyzed by the gasometric method. As controls, 5 cc. samples of the undiluted urines were analyzed

TABLE III.

Nitrogen Determination in Urine.

Urine No.	Gasometric micro-Kjeldahl.				Macro-Kjeldahl.
	Urine taken for analysis.	Pressure of N_2 at 2 cc. volume.	Temperature.	Nitrogen per 100 cc. urine.	Nitrogen per 100 cc. urine.
	cc.	*mm.*	*°C.*	*gm.*	*gm.*
1	0.200	261.4	22.0	0.414	0.422
		260.9	23.0	0.412	0.409
2	0.200	452.2	23.0	0.714	0.721
		451.4	23.0	0.713	0.728
3	0.200	202.4	23.0	0.320	0.321
		201.9	23.0	0.319	0.329
4	0.0667	233.9	23.5	1.105	1.144
		231.5	20.8	1.104	1.141
5	0.200	344.9	31.0	0.548	0.568
		347.3	21.5	0.552	0.569
6	0.0667	271.2	21.5	1.292	1.320
		276.3	22.0	1.317	1.312
		274.6	22.0	1.305	
7	0.100	329.7	22.5	1.043	1.060
		332.9	23.0	1.051	1.069
		331.9	23.0	1.048	

in duplicate by the usual Kjeldahl-Gunning method, each sample being digested with 20 cc. of sulfuric acid, 10 gm. of potassium sulfate, and a small crystal of copper sulfate. The results are given in Table III.

Analyses of Serum.

In order to ascertain the degree of constancy attainable in the analysis of a complex biological mixture five pairs of duplicate analyses were performed on one sample of horse serum. The serum was diluted to 10 volumes with 0.85 per cent salt solution. For each digestion 3 cc. of serum solution, equivalent to 0.3 cc. of

TABLE IV.

Repeated Analyses of One Specimen of Horse Serum.

No. of digest table.	Pressure at 2 cc. volume of N_2 from 0.12 cc. serum.	Temperature.	Nitrogen in sample.	Nitrogen per 100 cc. serum.
	mm.	°*C.*	*mg.*	*gm.*
1	426.9	25.0	1.337	1.114
	426.5	25.0	1.336	1.113
2	426.7	25.3	1.336	1.113
	425.5	25.3	1.333	1.110
3	422.7	25.3	1.324	1.103
	428.1	25.3	1.342	1.117
4	430.2	25.0	1.349	1.123
	426.1	24.0	1.341	1.117
5	427.9	24.0	1.346	1.122
	427.5	24.0	1.345	1.121
Macro-Kjeldahl.....				1.113
				1.117
				1.105

serum, were mixed with 2.5 cc. of the 1:3 phosphoric-sulfuric acid mixture and 2 gm. of potassium persulfate. The solutions cleared after about 5 minutes heating. Each digest was diluted, neutralized with 40 per cent NaOH, and finally brought to 25 cc. volume. Of this, 10 cc. portions, equivalent to 0.12 cc. of serum each, were transferred to the gas apparatus for determination of the ammonia. Deviations between the two results from each solution indicate the degree of variability due to the final hypobromite nitrogen determination in the gas apparatus, while the differences between the

different digests indicate the degree of error that may be introduced in the digestion process.

For the control determinations by macro-Kjeldahl 10 cc. portions of the 1:10 serum dilution were each digested with 20 cc. of sulfuric acid, 10 gm. of potassium sulfate, and a small crystal of copper sulfate. The ammonia was distilled into N/14 acid, of which it neutralized (after correction of 0.27 for blank) 11.13, 11.17, and 11.05 cc. in three determinations. The results are given in Table IV.

Of the ten gasometric determinations, nine gave between 1.333 and 1.349 mg. of nitrogen, maximum deviation being ± 0.5 per cent of the mean. The other result, 1.324 mg., deviated 1.2 per cent. The serum nitrogen content of 1.113 gm. per 100 cc. indicated by the average of the gasometric analyses agrees sufficiently well with the average of 1.108 gm. by the macro-Kjeldahl analyses to confirm the approximate correctness of the empirical 1.04 correction factor used in the calculation of the hypobromite results.

SUMMARY.

A micro-Kjeldahl method is described in which a quick digestion is obtained by the use of a mixture of sulfuric and phosphoric acids and potassium persulfate, and in which the ammonia formed is determined gasometrically. The neutralized digest is washed into the manometric gas apparatus of Van Slyke and Neill, and is there treated with hypobromite. The nitrogen gas is evolved in 2 minutes. Results are reproducible to within 1 per cent. The method obviates distillation and the use of standard solutions.

The greater part of the experimental work involved in developing the method here reported was carried out by John Plazin.

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COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

XI. FATE OF DIPHENYLACETIC ACID IN THE ANIMAL BODY.

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(Received for publication, November 1, 1926.)

In completing our series of experiments in the phenylacetic acid derivatives we thought it might be of interest to feed diphenylacetic acid to dogs and rabbits, also if found relatively harmless, to determine its fate in the human organism.

EXPERIMENTAL.

Diphenylacetic acid was fed by means of a stomach tube to rabbits as a water solution of the sodium salt. The substance proved to be slightly more toxic than phenylacetic acid so that a 1 gm. dose is about the average which can be well tolerated by a grown rabbit. The urine was collected in 24 hour periods. Total nitrogen, urea nitrogen, total sulfur, and ethereal sulfate were run in order to determine the effect on endogenous metabolism. Millon's test, ferric chloride test, and the reduction according to Benedict were always done. The urine was next evaporated on a water bath and extracted in the usual manner. After feeding 4 gm. of the acid to a rabbit, 1.5 gm. (37.5 per cent) of the substance were recovered unchanged. 2 gm. doses were fed to two rabbits and the urine collected for 48 hours. In this case 2.2 gm. (55 per cent) of the unchanged material were recovered. It was noted that there was always a very strong reduction with Benedict's solution after the feeding of diphenylacetic acid not only in fresh urine but in urine which had been evaporated nearly to dryness. In attempts to isolate this reducing substance, the ammonium sulfate method was first used. It proved a failure.

Ether extracts of the urine were found to be contaminated with hippuric acid and diphenylacetic acid, making a purification of the substance by means of fractional crystallization impossible. The following method was finally used: Concentrated lead acetate solution was added to the urine until no further precipitate filtered off, and the filtrate saturated with basic lead acetate solution. This caused a second heavy precipitation and brought down most of the reducing substance. After filtration by suction, the residue was repeatedly washed with cold water, when the lead was precipitated with hydrogen sulfide. The filtrate was evaporated *in vacuo* at a temperature of 10–12°. On concentration small amounts of diphenylacetic acid crystallized out and were filtered off. A dark syrupy mass of very high reducing power remained.

Diphenylacetylglucuronic Acid.

This substance was isolated from the syrupy mass described above by dissolving the mass in alcohol and then extracting in a separating funnel with large quantities of ether in a slightly acid solution. The ether solution thus obtained was kept in the ice box in a tightly stoppered bottle for a period of from 8 to 10 weeks. During this time there formed on the sides and bottom of the receptacle a deposit of a fine white amorphous powder which proved to be diphenylacetylglucuronic acid. The substance is easily soluble in water, absolute alcohol, acetone, benzene, carbon disulfide, and most of the organic solvents, while it is relatively soluble in cold ether. It decomposes at 180–185°. It is levorotatory in water solution, but like practically all the other glucuronic acid conjugation products it changes to a dextrorotation when in alkaline solutions. The substance reacts with all the reducing reagents without previous hydrolysis (Fehling's solution, Nylander's solution, Benedict's reagent, and even Barfoed's reagent). It forms no cyanhydrin nor does it appear to react with phenylhydrazine. Its ready reaction with all the reducing reagents without previous hydrolysis would tend to prove its instability in hot solutions, particularly when these are strongly alkaline. This would indicate that it belongs to the ester type of glucuronic acid conjugate rather than the ethereal or glucoside type and likely has the following graphical formula.

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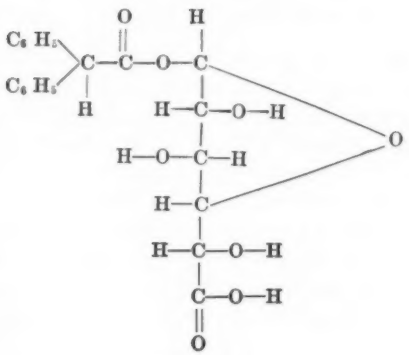

Analysis.

0.3236 gm. substance.	C 61.32,	61.55 per cent.
0.2214 " "	H 6.01,	5.45 " "
Calculated $C_{20}H_{20}O_8$.	C 61.82, H 5.15	" "

The 0.3 gm. of the substance was completely hydrolyzed by boiling for 30 minutes under a reflux condenser with 30 cc. of normal HCl, then neutralized with NaOH. The reducing power as calculated in terms of glucose was converted into glucuronic acid by the factor 1.085. Found, 0.1536 gm. of glucuronic acid or 102 per cent. 0.1220 gm. of the substance without hydrolysis indicated the presence of 0.058 and 0.059 gm. of glucuronic acid or 95 and 97 per cent of the theoretical yield, which indicates that practically complete hydrolysis took place during the process.

Specific Rotation.

The rotation of a 1 per cent solution of diphenylacetylglucuronic acid was:

$$[\alpha]_D^{20} = \frac{100 \times -1.39 \text{ per cent}}{2 \times 2} = -34.75°.$$

After feeding four rabbits 2 gm. each of diphenylacetic acid and collecting the urine for 24 hours each, the following amounts of glucuronic acid were obtained from the various urines: 0.1888, 0.1987, 0.1780, 0.2517 gm. as judged by reduction of the fresh urine with Benedict's reagent.

Diphenylacetylglycocoll.

As it was quite probable that we might encounter a conjugate of diphenylacetic acid with glycine in either the dog or rabbit, we decided to synthesize this product and study its properties.

10 gm. of diphenylacetic acid were heated with 70 gm. of thionyl chloride on an oil bath under a reflux condenser for 2 hours. The excess thionyl chloride was then distilled off. Under reduced pressure the chloride of diphenylacetic acid distilled over as a thick oily material. The crude material was used for the following synthesis; allowing for 8 gm. of acid chloride; 2 gm. of glycocoll were dissolved in hot water and this solution was made slightly alkaline

TABLE I.
Ether Extractions.

Amount fed.	Collection of urine.	Urine volume.	Glucuronic acid.	Diphenylacetic acid.	Per cent fed.
gm.	*hrs.*	*cc.*	*gm.*	*gm.*	
2.5	12	530	0.2371	0.2591	10.32
2	36	410	0.2607	0.2849	14.25
2	16	388	0.2929	0.3199	15.49
2	24	412	0.3546	0.3872	19.36
2	24	340	0.2567	0.2803	14.02
6	60	1310	1.8976	2.0722	34.54

with normal NaOH. The acid chloride was added gradually with constant shaking in the course of 3 hours, and gentle heat was used to aid the reaction while the mixture was kept slightly alkaline. Contents of the flask were filtered off while warm and the filtrate acidified to Congo red. It was again filtered to separate out the free diphenylacetic acid. The diphenylacetylglycocoll was allowed to crystallize slowly from the filtrate. Diphenylacetylglycocoll is a white solid crystallizing from water in prisms, soluble in ether, water, and ethyl alcohol; insoluble in chloroform and benzene. M. p. 157°C.

Kjeldahl Analysis for Nitrogen.

0.2833 gm. subtance required 10.42 cc. 0.1 N acid 5.15 per cent N.
0.2066 " " " 7.67 " 0.1 " " 5.24 " " "
Calculated for $C_{16}H_{13}O_3N$. N, 5.20.

Micro Analysis for Hydrogen and Carbon.

11.66	mg.	substance	gave	5.855	mg.	H_2O.	5.58	per cent	H.
10.00	"	"	"	5.022	"	"	5.69	"	"	"
5.322	"	"	"	13.92	"	CO_2.	71.32	"	"	C.
8.112	"	"	"	21.096	"	"	70.92	"	"	"

Calculated. H 5.58, C 71.38.

Fate of Diphenylacetic Acid in the Dog.

6 gm. of diphenylacetic acid were fed to a dog of 20 kilos in two 3 gm. doses. Urine was collected for 48 hours after the last dose. It was treated as usual. From the ethyl acetate extraction of the urine 4.4 gm. were recovered or 73.3 per cent of the unchanged substance. The ether extracts of various subsequent dog urines after feeding diphenylacetic acid yielded a glucuronic conjugate as shown by Table I.

Fate of Diphenylacetic Acid in Man.

Five 10 gm. doses of diphenylacetic acid were ingested by various individuals in the course of 24 hours. The urine was treated in the usual manner described above. There was no sign of a glutamine or a glycine conjugate. Only the same glucuronic acid compound was found which we had met in experimenting with dogs and rabbits.

SUMMARY AND CONCLUSIONS.

Diphenylacetic acid was fed to rabbits and dogs, and ingested by human beings in amounts sufficient to follow its metabolic fate. No glycocoll or glutamine conjugate was found in any instance. Most of the acid was excreted unchanged. The only conjugation found was a glucuronic acid conjugation present in the urine of the human subject as well as the animal. Diphenylacetylglycine was prepared synthetically. Diphenylacetylglucuronic acid was isolated and its properties studied.

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IRON IN NUTRITION.

III. THE EFFECTS OF DIET ON THE IRON CONTENT OF MILK.*

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(Received for publication, November 10, 1926.)

The fact that the iron content of milk is relatively low and that milk is the sole food of young mammals during a considerable period of rapid growth, makes the study of the relation of diet to the iron content of milk of practical importance. The familiar experiments of Bunge and Abderhalden showed that the ash of milk and the ash of the new born animal, of the same species, were very similar in all constituents except iron, which is almost six times as abundant in the ash of the young animal as it is in the ash of the milk on which it is nourished. Nature has compensated for this difference by supplying the animal at birth with a reserve store of iron, but often this supply is depleted before food richer in iron supplements the milk. If the iron content of the mother's milk could be increased by giving her a varied diet, some of the difficulties experienced with whole milk diets might be prevented.

Sherman (1), reviewing the literature on this problem, finds varied results and conclusions. Some investigators claim to have been able to increase the percentage of iron in milk by feeding different compounds of iron, while others have arrived at the opposite conclusion. Much of this was early work and the methods for Fe determination were still crude. Recent work on this problem is very meager; in fact, the results which are recorded in the recent literature were obtained from experiments which did not have this specific problem in mind. Hess, Unger, and Supplee (2), in studying the antiscorbutic potency of milk produced by cows

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getting different rations, give analyses which show the iron content of milk produced by cows on pasture to be twice as high as that from cows on a normal winter ration. These workers do not give the methods used for iron determination, nor do they make any comment on the variations found.

The increasing amount of work on iron metabolism readily shows the necessity of studying the iron content of milk produced under different conditions. In studies on iron metabolism the basal ration of the experimental animals generally consists of whole milk, presumably cow's milk. If the percentage of iron in the milk of these cows fluctuates with the change in their diet, then any variation in their ration may influence the results. On the other hand in studying changes in the young due to variation in diet, it is important to know if these changes have been caused by fluctuations in the amount of iron in the mother's milk. McGowan and Crichton (3), in their paper on "Iron Deficiency in Pigs" realized the value of studying the amount of iron in the mother's milk. They were unable to determine whether the young pigs, from sows receiving daily administrations of Fe_2O_3, were benefited by the transfer of this iron through the milk, or by obtaining an extra supply from refuse.

We became interested some time ago in studying this problem further by actually determining the iron in milk from animals receiving a basal ration plus various additions, but we were handicapped by the lack of accurate methods of determining iron. Recently we developed a method (4) by which the amount of iron in milk may be determined very accurately. Making use of this method we have studied the iron content of milk from goats receiving different rations. The two goats used were mature lactating animals which were brought into the laboratory and kept on a basal ration 1 week before the experiment was begun. The basal ration consisted of 2 parts of a grain mixture and 1 part of alfalfa hay. The approximate daily intake was 300 gm. of hay and 600 gm. of the grain mixture per goat. The grain mixture consisted of ground yellow corn 59 parts, wheat bran 25 parts, oil meal 15 parts, and NaCl 1 part.

After 1 week of preliminary feeding, the animals were placed on the basal ration for a period of 13 weeks. They were supplied with distilled water *ad libitum*. During this time the animals

were milked twice daily; at each milking the milk was carefully measured and an aliquot taken for a weekly composite sample. The milk was collected in glass vessels and the samples kept in glass bottles to prevent any possible contamination with iron.

TABLE I.

Record of Yield of Milk and Percentage of Iron Found.

Week.	Amount of milk.	Percentage of iron.	Amount of milk.	Percentage of iron.
	Goat 1.		Goat 2.	
	Period 1. Basal ration.			
	gm.		*gm.*	
1	7460	0.000188	7015	0.000177
2	7230	0.000178	5400	0.000175
3	5960	0.000174	3980	0.000166
	Average.....	0.000180	Average.....	0.000172
	Period 2. Basal ration + 0.4 gm. of Fe_2O_3 daily.			
4	5150	0.000170	4700	0.000175
5	4780	0.000186	4480	0.000156
6	3750	0.000190	3940	0.000186
7	2470	0.000183	4620	0.000163
8	2190	0.000175	4070	0.000156
	Average.....	0.000181	Average.....	0.000167
	Period 3. Basal ration + 0.4 gm. of Fe_2O_3 + 450 gm. of fresh cabbage daily.			
9	2110	0.000158	3280	0.000156
10	1730	0.000155	2980	0.000162
11	1410	0.000176	2540	0.000142
12	1085	0.000164	2075	0.000156
13	525	0.000160	2810	0.000157
	Average.....	0.000163	Average.....	0.000155

The composite samples were preserved with formaldehyde. Each sample was analyzed for its iron content by the afore mentioned method (4). The 13 weeks were divided into three periods: the first period, consisted of 3 weeks, during which the animals were

fed the basal ration with no additions; in the second period, consisting of 5 weeks, they were fed the basal ration plus 0.4 gm. of Fe_2O_3 daily (per animal); and in the third period of 5 weeks, they received the basal ration plus 0.4 gm. of Fe_2O_3, plus 450 gm. of fresh cabbage daily (per animal).

The daily addition of 0.4 gm. of Fe_2O_3 increased the iron intake of each animal fivefold above the intake on the basal ration. The use of fresh cabbage, in Period 3, was suggested to us by our earlier

TABLE II.

Record of Rate of Anemia Production in Rabbits on Different Milks.

Milk from period No.	Rabbit No.	Average decrease in hemoglobin per week.	Average decrease in erythrocytes per week.	Rabbit No.	Average decrease in hemoglobin per week.	Average decrease in erythrocytes per week.
	Goat 1.			Goat 2.		
		per cent			*per cent*	
1	190	13.02	500,000	192	10.78	700,000
	191	6.02	240,000	193	9.94	600,000
	Average..	9.52	370,000	Average..	10.36	650,000
2	195	6.30	200,000	197	6.30	600,000
	196	10.60	900,000	198	2.40	100,000
	Average..	8.45	550,000	Average..	4.35	350,000
3	276	8.70	300,000	278	11.40	500,000
	277	7.10	300,000	279	7.10	700,000
	Average..	7.90	300,000	Average..	9.25	600,000

work (5) which showed the beneficial effect of the direct feeding of green cabbage on the assimilation of iron in young rabbits.

Table I shows the amount of milk given by each goat and the percentage of iron in the milk from each of the goats during the various feeding periods.

In order to make a further study of any possible change in the milk during the three feeding periods, two rabbits were started when 1 month old on the milk from each goat during each individual period. We have shown before that by placing a young rabbit at weaning time on a diet of cow's milk alone the percentage of

hemoglobin decreases from about 95 per cent to 55 per cent and the erythrocyte count decreases from about 6 million to 3.5 million per c.mm. in a period of 5 weeks. We therefore could follow the rate at which the blood constituents would change when the rabbits were receiving the different milks. The rabbits were kept on the specific milks from 3 to 5 weeks and the percentage of hemoglobin and the erythrocyte count made weekly by our usual methods. From these data we have calculated the average decrease in hemoglobin and erythrocyte count per week for each animal during the different periods. These data are recorded in Table II.

From Table I it may readily be seen that the iron content of milk *is not increased* by feeding large amounts of iron in the ration or even by feeding green material such as cabbage in addition to the added iron. The percentage of iron in the milk seems to be an established amount for *any* specific animal and cannot be varied even by drastic changes in the iron content of its diet.

Table II shows that feeding large amounts of Fe_2O_3 or the oxide with green cabbage brings about no change in the milk which will prevent a young growing animal from developing nutritional anemia. The rabbits receiving milk from the goats when they were fed the iron or the iron plus the cabbage developed anemia as rapidly as the rabbits fed the milk from the goats while on the basal ration. Of course we cannot say that feeding green material has no quantitative influence upon any factors in milk which have to do with iron assimilation. All we can say is that if there were such changes they were not of sufficient magnitude to show any influence when feeding such low levels of iron as found in milk. If iron had been added to the milk when fed to the rabbits some influence might have been noted, but this was not done.

After it was found that Fe_2O_3 had no effect on the iron content of milk we decided that it would be valuable to try some iron salt which is more soluble than the oxide. We, therefore, conducted a second experiment using two other goats. These animals, after the preliminary feeding, were placed on the same basal ration as used in the first experiment. For the first period of 3 weeks they were given only the basal ration; the second period of 4 weeks the iron content was increased fivefold by the addition of 1.33 gm. of $FeSO_4 \cdot 7H_2O$ per individual daily. The milk was

collected and analyzed in the same manner as before. The results of this experiment are given in Table III.

It is apparent that even by the use of a very soluble iron salt such as $FeSO_4$ no change in the iron content of the milk could be produced.

Further evidence substantiating our results was obtained by the analysis of milk from cows on different rations. For this purpose

TABLE III.

Record of Yield of Milk and Percentage of Iron Found.

Week.	Amount of milk.	Percentage of iron.	Amount of milk.	Percentage of iron.
	Goat 3.		Goat 4.	
	Period 1. Basal ration.			
	gm.		*gm.*	
1	4760	0.000212	3345	0.000227
2	4610	0.000211	3490	0.000209
3	4290	0.000179	3290	0.000226
	Average.....	0.000200	Average.....	0.000220
	Period 2. Basal ration + 1.33 gm. of $FeSO_4 \cdot 7H_2O$ daily.			
4	3920	0.000215	3140	0.000236
5	3965	0.000213	3450	0.000222
6	3945	0.000200	3355	0.000223
7	3965	0.000193	3095	0.000216
	Average.....	0.000205	Average.....	0.000224

we used six cows all getting the same grain mixture but three of the animals were fed alfalfa hay as roughage and three timothy hay. The cows fed alfalfa hay were also receiving about 150 cc. of cod liver oil daily. This gave two groups of animals receiving rations with a considerably different iron intake, especially when one notes that the alfalfa hay contained 0.0151 per cent Fe and the timothy hay 0.0071 per cent Fe. The milk was collected directly from the udder in glass vessels to prevent contact with any material which might affect the iron content, and analyzed by our usual method. We also noted the length of lactation to allow any

possible correlation between the length of time of milking and the iron content. The results are given in Table IV.

We find that the average per cent of iron in the milk from the two periods is very similar and that the milk from the cows receiving the ration with the higher iron intake was no higher than that on the lower level of iron intake. However, it is very significant to note that the percentage of iron in the milk from the individual cows varies greatly. This may explain some of the erroneous results obtained by earlier workers. There seems to be no relation between the amount of iron in the milk and the length of lactation.

TABLE IV.

Iron Content in Milk from Cows on Different Rations.

Cow No.	Kind of roughage.	Percentage of iron in milk.	Length of lactation.
			days
11	Alfalfa hay.	0.000187	150
12	" "	0.000367	72
20	" "	0.000204	111
	Average.....	0.000234	
24	Timothy hay.	0.000213	148
25	" "	0.000238	101
26	" "	0.000288	175
	Average.....	0.000246	

CONCLUSION.

1. The iron content of goat's milk cannot be increased by feeding the goats Fe_2O or the very soluble iron salt $FeSO_4 \cdot 7H_2O$. No noticeable change in iron content can be detected even if the addition of the iron increases the original iron content of the ration fivefold. Feeding fresh green cabbage together with the Fe_2O_3 also had a negative effect on increasing the amount of iron in the milk.

2. The addition of Fe_2O_3 or Fe_2O_3 plus fresh green cabbage to the ration of a goat brought about no changes in the milk which would prevent the development of nutritional anemia when fed to young growing rabbits.

3. There was no difference in the average iron content of milk from cows on such different rations as alfalfa hay and timothy hay. The alfalfa hay contained twice as much iron as the timothy hay. The percentage of iron in the milk from individual cows varied as much as 100 per cent. This variation cannot be correlated with the length of the lactation period.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

VIII. THE CALCIUM LEVEL AND SUNLIGHT AS AFFECTING CALCIUM EQUILIBRIUM IN MILKING COWS.*

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In a recent publication (1) in this series, we showed that it was impossible to maintain a positive calcium balance in cows milking 45 to 60 pounds per day where the ration consisted of a grain-grain by product mixture, corn silage, and green grasses. It made little difference in respect to the calcium balances whether the metabolism experiment was conducted away from direct sunlight or in the direct sunlight of May and June. The degree of calcium negativity did seem to be somewhat reduced when the cows were in direct sunlight as compared with indoors, but in no case was a negative balance changed to a positive one. We left open the question whether light has any important influence upon the calcium and phosphorus metabolism of dairy cows. This particular question is still under investigation and experiments are in progress which we hope will give a definite answer to this highly important problem. In the meantime work in this laboratory has been in progress upon the question of the influence of light—sunlight and the emanations from a quartz mercury lamp—upon the antirachitic potency of cow's milk. The results of these researches will appear in a separate publication.

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Since the lime intake of a green grass-silage-grain mixture is comparatively low, it was considered important that these earlier experiments be repeated in sunlight and out of sunlight, but with a calcium oxide intake considerably increased. The green grasses used in our earlier experiments were lawn clippings and consisted mainly of a mixture of June grass and white clover with the former predominating. The average calcium oxide content of these mixed grasses calculated to the dry basis was approximately 1.04 per cent CaO. The total calcium oxide content of the ration of grains, silage, and grasses calculated to a dry basis was approximately 0.52 per cent, or an intake of 70 to 80 gm. of calcium oxide daily. This was certainly not a liberal supply of calcium in view of the fact that calcium is liberally secreted into milk, constituting approximately 2.5 per cent of the dry solids as CaO, and in view of the additional fact that the antirachitic potency of the feeds used was not of a high order.

In the maintenance of calcium equilibrium in a milking animal it is apparent that the following facts must always be given consideration: first, the calcium level of the ration; second, the antirachitic potency of the feeds; third, the volume of milk produced; fourth, the physiological status of the animal which may influence calcium absorption from the intestine. Apparently, as first pointed out by Meigs (2), this last factor may become at times an important factor.

EXPERIMENTAL.

We again planned the experiments to include a metabolism period of 4 weeks indoors and away from the influence of direct sunlight and another period of 3 weeks in direct sunlight. The additional feature of the experiment was an increase in the calcium level of the ration through the feeding of ½ pound of marl ($CaCO_3$) daily to each individual. This made the per cent of CaO in the ration calculated to a dry basis approximately 1.50 per cent as contrasted with 0.52 per cent when the ration was unsupplemented. The daily ration consisted of 30 pounds of corn silage, 40 pounds of green grasses, and a grain mixture consisting of 59 parts of yellow corn, 25 parts of wheat bran, 15 parts of oil meal, and 1 part of common salt. The amount of grain allowed daily was regulated by the milk flow and was fed at the rate of 1 pound for 3 to 4 pounds of milk produced.

The lawn clippings were cut daily from 1 to 4 p.m., divided for the evening and morning ration, and sampled daily for analysis. The marl was a commercial product containing 48.5 per cent CaO. It was mixed with the grain. A soft water containing 2 gm. of CaO per 110 pounds was used.

We planned to use three liberally milking cows for the active metabolism work. Early in January, 1926, six cows were set aside and fed a ration of corn silage, alfalfa hay of unknown curing history, and a grain-grain by product mixture. They were always kept indoors. After freshening, a timothy hay of unknown curing history was substituted for the alfalfa hay. This was done to lower the calcium intake. The three cows that were finally used in the metabolism experiment were pure bred Holsteins milking from 45 to 60 pounds of milk daily during the metabolism work. Cow 1 was 6 years old and freshened in February, 1926. It gave no trouble in food consumption. It weighed 1442 pounds at the beginning of the experiment, May 17, and 1468 pounds at the termination of the work, July 5. Cow 2 was 4 years old and freshened in April, 1926. It was nervous and excitable in the metabolism stall, failed on occasions to consume the ration completely, and, as shown in Table II, gave us a record at variance with the records of the other two animals. We are recording the data from this cow as it appears of importance in emphasizing the necessity for the choice of older or, at least, quiet animals with phlegmatic temperaments for such studies as are here involved. This animal weighed 1203 pounds at the beginning of the experiment and 1222 pounds at the end. Cow 3 was 4 years old and freshened in March, 1926. Although but 4 years old, it was of quiet disposition and gave no trouble in food consumption. Its weight at the beginning of the experiment, May 17, was 1308 pounds and 1380 pounds on July 5, when the experiment ended.

Quantitative collection of all excreta was made by men working in 8 hour shifts and quantitative analysis for calcium of all ingo and outgo recorded. The green plant tissue was sampled daily and analyzed. The corn silage was sampled a number of times during the course of the experiment for its lime content.

As mentioned above, the first period of the metabolism work consisted of 4 weeks duration and was conducted indoors and out

of contact with direct sunlight. In the second period of 3 weeks the experiment was conducted in direct sunlight. The details of

TABLE I.

Calcium Balance and Milk Production of Cow 1.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass-no sunlight period.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
May 17–24	1194.33	298.82	1.90	1495.05	1368.84	−127.21	374
" 24–31	971.56	293.08	3.26	1267.90	1359.80	+91.90	362
June 1–7	966.90	292.68	2.85	1262.22	1332.72	+70.50	362
" 7–14	1069.44	287.92	0.92	1358.28	1402.32	+44.04	358
Green grass-sunlight period.							
June 14–21	977.98	280.47	4.31	1262.76	1371.11	+108.35	351
" 21–28	1053.36	249.97	2.33	1305.66	1453.68	+148.02	359
" 28–July 5	1047.94	286.16	2.91	1337.01	1436.77	+99.76	368

TABLE II.

Calcium Balance and Milk Production of Cow 2.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass-no sunlight period.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	lbs.
May 17–24	1131.61	301.44	1.85	1434.90	1360.63	−74.27	386
" 24–31	1146.54	340.75	1.67	1488.96	1351.53	−137.43	408
June 1–7	1070.16	331.28	10.10	1411.54	1324.45	−87.09	400
" 7–14	1125.72	330.86	9.01	1465.59	1394.15	−71.44	398
Green grass-sunlight period.							
June 14–21	996.48	312.79	15.33	1324.60	1362.84	+38.24	376
" 21–28	908.28	263.09	1.86	1173.93	1445.41*	+271.58	336
" 28–July 5	1123.08	322.87	11.08	1457.03	1428.50	−28.53	370

* Calculated and not the actual intake. May 25 to 27, appeared nervous; slow in consuming feed. June 22 to 24, off feed; consumption low. June 24 and thereafter, consumption normal.

arrangement of stalls and method of conducting such an experiment have already been described in a former publication (1).

During the sunlight period, which lasted from June 14 to July 5, the average daily sunlight exposure amounted to 4.8 hours per day. In Tables I, II, and III are shown the results of these experiments as conducted out of sunlight and in direct sunlight.

DISCUSSION.

Viewed as a whole, the results secured indicate that equilibrium or a positive calcium balance can be secured with liberally milking cows receiving a ration of grains, grain by products, corn silage, and green grasses only when that ration is supplemented with lime. Without this supplement, as we have previously shown, with-

TABLE III.

Calcium Balance and Milk Production of Cow 3.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Green grass-no sunlight period.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*lbs.*
May 17–24	1037.18	219.13	14.02	1270.33	1364.50	+94.17	309
" 24–31	1018.77	242.45	8.83	1270.05	1351.53	+81.48	306
June 1–7	1022.20	204.66	25.54	1252.40	1324.45	+72.05	304
" 7–14	1084.41	228.50	26.70	1339.61	1394.15	+54.44	299
Green grass-sunlight period.							
June 14–21	1055.23	238.49	22.28	1316.00	1362.84	+46.84	298
" 21–28	1149.81	250.78	27.57	1428.16	1445.41	+17.25	315
" 28–July 5	1081.82	234.52	13.58	1329.92	1428.50	+98.58	307

drawal of lime from the skeleton will follow and negative balances ensue. The unsupplemented ration used in previous experiments and in the experiments reported in this paper was an imitation of the summer feeding of many dairy cows, and it is apparent from the data obtained that milking cows so treated and fed must be in negative lime balance. By supplementing such a ration with lime to bring the level of CaO intake to approximately 1.50 per cent of the dry ration, or a daily total intake of about 200 gm. of calcium oxide per day, equilibrium or positive balances could be maintained whether the cows were indoors or in direct sunlight. Since positive calcium balances in two of the experimental animals

were secured in the first period (no sunlight period) and were continued in the sunlight period, it is manifest that no statement can be deduced from such data in respect to the influence of sunlight on the calcium and phosphorus metabolism of dairy cows. In our previous experiment (1) on the influence of sunlight on the calcium metabolism of milking animals, there did seem to be some reduction in the lime losses from the body during the sunlight period but negative balances were not changed to positive ones. Further work with more intensive ultra-violet light illumination must be done before a definite answer can be given to the problem of whether light will influence the calcium and phosphorus metabolism of the dairy cow.

The data do, however, fully support the view that with the mild antirachitic properties secured from such feed as green grasses cut in the afternoon and supplemented with corn silage and grains, the calcium requirement of a cow milking 45 to 60 pounds per day can be met when the daily lime intake is in the neighborhood of 200 gm. This, of course, includes the calcium oxide of the feed as well as the supplement. The data further warrant the statement that supplementing a ration of ordinary summer pasture (mainly non-legume) with lime carriers will be necessary for liberally milking cows if the maintenance of calcium equilibrium is desired.

The fact that by raising the calcium level of intake calcium equilibrium could be secured with the ration used, finds its parallel in the results that have been secured on phosphorus metabolism. In most of our experimental work (3) with cows in which a liberal phosphorus intake has been secured through the feeding of grains supplemented with wheat bran, equilibrium or positive balances in respect to phosphorus have been obtained even when the experiment was conducted indoors and out of contact with direct sunlight. The phosphorus intake expressed as P_2O_5 has been approximately 200 gm. per day for cows milking 45 to 60 pounds per day.

The deductions made in this paper are from the records of Cows 1 and 3. These animals were quiet and comfortable throughout the experiment and their feed consumption was complete. Cow 2 gave a record showing negative calcium balances during the entire first period (no sunlight) and a positive balance in 2 of the weeks

of the sunlight period. These positive balances in the sunlight period are to be attributed to a calculated intake and not an actual intake—the food residues not being analyzed. These results are not to be interpreted as indicating a positive effect of sunlight. We believe that the negative calcium balance shown by this animal during the first period was due to its nervous, excitable condition which influenced calcium absorption and that its record does not represent a normal record.

SUMMARY.

1. On a daily ration of 40 pounds of green grasses, 30 pounds of corn silage, and 12 to 14 pounds of grain and grain by products, the calcium oxide intake for a cow milking 45 to 60 pounds daily was about 70 to 80 gm. Calcium equilibrium cannot be maintained on that level of intake either in or out of sunlight. By increasing the calcium level to approximately 200 gm. of CaO per day, calcium equilibrium was established whether the metabolism experiment was conducted indoors and out of direct sunlight or in direct sunlight.

2. Apparently there was sufficient of the antirachitic factor in the green grasses cut from 1 to 5 p.m. and the remainder of the ration to maintain calcium equilibrium when the intake of calcium was at the higher level.

3. These data would support the view that it is desirable to supplement the ordinary summer pasture (non-legume) ration of liberally milking cows with some lime carrier. Our experimental animals received ½ pound of marl (calcium carbonate) per day.

4. Since calcium equilibrium was established out of direct sunlight by the use of the higher level of calcium intake, our data contribute no additional facts to the question of the influence of ultra-violet light on the calcium and phosphorus metabolism of dairy cows.

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DIETARY FACTORS INFLUENCING CALCIUM ASSIMILATION.

IX. FURTHER OBSERVATIONS ON THE INFLUENCE OF COD LIVER OIL ON CALCIUM ASSIMILATION IN LACTATING ANIMALS.*

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In 1921 we published data (1) showing that the administration of cod liver oil to milking or dry goats changed negative calcium balances to positive balances. The experiments were of rather short duration and invariably led to impaired appetite, making the data less conclusive than we wished. With the development of our knowledge that the antirachitic factor is resident in the non-saponifiable matter of cod liver oil, it became apparent that a better method than feeding the raw cod liver oil was available for further study of the influence of this material on the calcium balances of milking goats. Consequently we planned to bring lactating goats into negative lime balance on a suitable ration and then administer the non-saponifiable matter of raw cod liver oil put up in gelatin capsules. In this way we believed that we could administer the antirachitic factor for a long time without impairment of appetite and at the same time feed the equivalent of the raw cod liver oil in relatively high concentrations.

It is extremely important that it be definitely established whether raw cod liver oil will function in improving calcium assimilation in the lactating animal as has been established for the growing animal. *A priori* there seems to be no good reason why this should not be the case. However, Meigs and his associates (2) have recently published data showing in relatively short experi-

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ments that the administration of cod liver oil to the milking cow impaired the assimilation of calcium rather than improved it. They also state that in the case of one animal the appetite was interfered with after the administration of the cod liver oil. With the sudden administration of cod liver oil and the relatively short experimental period of a metabolism experiment, this is not surprising. We experienced the same situation with milking goats after a few weeks of cod liver oil feeding. With disturbed appetites will come decreased food intake, and with the delayed lag in intestinal excretion larger negative calcium balances will follow. Such data, however, will not give a true estimate in respect to the efficacy of raw cod liver oil, where it is gradually administered and where its administration is continued over a considerable span of the animal's life, thus accustoming the individual to its continued use. It is already established through production records (3), but not by metabolism experiments, that the administration of raw cod liver oil to laying hens is very effective in increasing egg production, and one is certainly led to believe that this phenomenon is associated with the antirachitic factor of cod liver oil and better calcium assimilation since the same result is secured through the application of ultra-violet light.

It is apparent that in milk production, as in egg production, the antirachitic factor will play an important part in the health of the animal if not in its producing capacity. Should further research fully establish the fact that artificially produced ultra-violet light or sunlight cannot directly furnish the antirachitic factor to certain important milk-producing animals, as for example the dairy cow, then recourse must be made to other available materials richly endowed with vitamin D. Among such materials for the present at least is cod liver oil, and consequently its rôle in the nutrition of the lactating animal must be fully understood.

EXPERIMENTAL.

For these experiments milking *goats* were again used. They were common American grades with no distinct breeding. They were brought to our metabolism room and usually placed in an open pen for at least a week before being placed in the metabolism cage. Quantitative collection of the excreta was made. The goats were milked twice daily. Analyses were applied to the

weekly collection of feces and to the weekly composite of aliquots of urine and milk taken daily. Calcium determinations in the feeds, milk, and feces were made by the McCrudden method after ashing. In the urine the calcium determination was made directly and without ashing as further suggested by McCrudden.

The ration used throughout was one made of chopped commercial alfalfa hay of unknown curing history and a grain mixture composed of 59 parts of yellow corn, 25 of wheat bran, 15 of oil meal, and 1 of common salt. The ration was fed in the proportion of 2 parts of the grain mixture to 1 part of chopped alfalfa hay which were mixed at feeding time. This made the alfalfa 33.3 per cent of the ration. On this ration negative lime balances were invariably and quickly established with milking goats. After a preliminary period of feeding the basal ration for 2 to 4 weeks, the non-saponifiable residue from cod liver oil was administered in gelatin capsules at rates equivalent to as high as 40 or 80 cc. of cod liver oil per day per individual.

The cod liver oil was saponified by boiling 280 cc. of the oil in 500 cc. of 20 per cent alcoholic KOH, cooling, and diluting with 2000 to 3000 cc. of water. The mass was then extracted with ether, two extractions of about 1500 cc. each being made. The ether extract was evaporated before the fan, and this residue from 280 cc. of the raw oil usually amounted to about $1\frac{1}{2}$ gm. This residue was diluted with dextrin to a weight of 3.5 gm., then divided into seven parts (one for each day of the week), and was equivalent to approximately 40 cc. of the raw oil. Each part was packed in a gelatin capsule and administered by opening the mouth of the animal, placing the capsule in the back of the mouth, and following with a drench of water. After a time the use of the water drench was found unnecessary. Administering cod liver oil in this way appeared to have no adverse effect upon the appetite. In some cases, as can be seen by consulting the tables, the equivalent of a daily administration of 80 cc. of cod liver oil was made. Further, in some cases the cod liver oil was saponified twice to reduce as much as possible the content of obnoxious material in the non-saponifiable residue.

As the work progressed, it became evident, as can be seen from Tables I, II, and III, that the administration of the non-saponifiable fraction of raw cod liver oil in capsules was having no favor-

able influence on the absorption of calcium. The appetite of the animal was not disturbed but assimilation was not improved. It occurred to us that because of the probable nature of vitamin D, its absorption from the digestive tract would be extremely

TABLE I.

Record of the Calcium Balance and Milk Production of Animal 1.

Period.	CaO in feces.	CaO in urine.	CaO in milk.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
			Period 1. No addition.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Jan. 7–14	39.7	0.24	5.3	45.2	36.9	−8.3	2897
" 14–21	40.7	0.40	5.3	46.4	43.1	−3.3	2920
" 21–28	49.3	0.45	6.0	55.7	43.1	−12.6	3416
Period 2. Non-saponified fraction equivalent to 40 cc. of cod liver oil daily. Saponified twice. Fed in capsules.							
Jan. 28–Feb. 4	41.0	0.26	6.4	47.6	43.1	−4.5	3289
Feb. 4–11	44.1	0.41	7.8	52.3	43.1	−9.2	4006
" 11–18	42.2	0.48	8.1	50.7	44.7	−6.0	4457
" 18–25	52.1	0.41	6.7	59.2	44.7	−14.5	3844
Period 3. Non-saponified fraction equivalent to 80 cc. of cod liver oil daily. Saponified once. Fed in capsules.							
Feb. 25–Mar. 4	59.4	0.51	7.8	67.7	44.7	−23.0	4103
Mar. 4–11	63.0	0.39	9.1	72.4	44.7	−27.7	4488
" 11–18	60.2	0.13	9.6	69.9	44.7	−25.2	4424
Period 4. Non-saponified fraction. Fed after solution in 15 cc. of corn oil; equivalent to 80 cc. of cod liver oil daily. Saponified once.							
Mar. 18–25	52.8	0.19	9.0	61.9	56.7	−5.2	4458
" 25–Apr. 1	45.1	0.46	8.6	54.2	44.2	−10.0	4178
Apr. 1–8	37.8	1.14	8.7	47.7	45.3	−2.4	3995
" 8–15	39.3	1.32	5.5	46.1	39.0	−7.1	2702
" 15–22	45.6	1.00	7.3	53.9	45.2	−8.7	3702
" 22–29	40.0	1.19	7.3	48.6	45.3	−3.2	3385

slow or practically nil unless it were administered in solution. Consequently we decided to administer the non-saponifiable part of cod liver oil not in capsules in the solid state but in solution in refined corn oil. By doing this we would escape the nauseating

effect of cod liver oil and yet administer the antirachitic factor in solution. The non-saponifiable matter equivalent to 40 or 80 cc. of raw cod liver oil was dissolved in 15 cc. of refined corn oil by gently warming on the water bath, and then mixed with the grain portion of the ration. When this was done a marked improvement in calcium assimilation was effected as can be seen from inspection of Tables I, II, and III.

TABLE II.

Record of Calcium Balance and Milk Production of Animal 2.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Period 1. No addition.							
Feb. 11–18	45.6	7.0	0.36	52.9	43.6	−9.3	3615
" 18–25	51.7	6.9	0.30	58.9	43.2	−15.7	3203
Period 2. Non-saponified fraction equivalent to 40 cc. of cod liver oil daily. Saponified once. Fed in capsules.							
Feb. 25–Mar. 4	53.1	7.0	0.41	60.5	43.6	−16.9	3402
Mar. 4–11	57.2	7.8	0.26	65.2	43.6	−21.6	3577
" 11–18	55.6	7.8	0.13	63.5	43.6	−19.9	3478
Period 3. Non-saponified fraction. Fed after solution in 15 cc. of corn oil; equivalent to 40 cc. of cod liver oil daily. Saponified once.							
Mar. 18–25	50.3	7.3	0.42	58.0	55.6	−2.4	3418
" 25–Apr. 1	33.1	7.0	0.55	40.6	42.7	+2.1	3027
Apr. 1–8	31.6	5.4	0.41	37.4	38.7	+1.3	2214

A study of the metabolism record of Animal 1, Table I, shows a distinct negative calcium balance in the preliminary period when only the basal ration was fed. In the second period the non-saponifiable fraction of cod liver oil put up in capsules and fed equivalent to a daily intake of 40 cc. gave no improvement in the calcium balance. When the non-saponifiable matter equivalent to 80 cc. of oil was fed daily (see Period 3) there was still no improvement in calcium assimilation. In fact, the greatest negative calcium balance was experienced in this period. When, however, the non-saponifiable material of 80 cc. of oil was redissolved in

15 cc. of refined corn oil and then administered, a marked improvement in calcium assimilation resulted (see Period 4). Positive calcium balances were not established but the fecal calcium was reduced from 60 gm. to approximately 40 gm. This animal had been in the metabolism cage for 3 months before the period of feeding the non-saponifiable fraction dissolved in corn oil, and it is not surprising that actual positive calcium balances were not established.

TABLE III.

Record of Calcium Balance and Milk Production of Animal 3.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Period 1. No addition.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Feb. 11–18	41.3	8.0	0.10	49.4	43.6	−5.8	3765
" 18–25	52.1	8.4	0.17	60.6	43.6	−17.0	3656
Period 2. Non-saponified fraction; equivalent to 80 cc. of cod liver oil daily. Saponified once. Fed in capsules.							
Feb. 25–Mar. 4	55.3	8.9	0.26	64.4	43.6	−20.8	4089
Mar. 4–11	55.0	8.1	0.18	63.2	43.6	−19.6	3437
" 11–18	61.1	7.1	0.15	68.3	43.6	−24.7	3378
Period 3. Non-saponified fraction. Fed after solution in 15 cc. of corn oil. Equivalent to 80 cc. of cod liver oil daily. Saponified once.							
Mar. 18–25	49.0	6.7	0.20	55.9	55.6	−0.3	3315
" 25–Apr. 1	35.0	6.2	0.28	41.4	43.1	+1.7	3002
Apr. 1–8	30.1	6.4	0.79	37.4	44.1	+6.7	3081
" 8–15	35.4	6.3	0.91	42.6	44.1	+1.5	2950
" 15–22	33.9	6.3	0.36	40.6	44.1	+3.4	3100

The record of Animal 2, Table II, is still more convincing than the record of Animal 1. Improvement in calcium assimilation was not secured through the feeding of the dry, pasty non-saponifiable fraction of cod liver oil; but when this material was redissolved in 15 cc. of corn oil, a marked improvement followed. The fecal calcium output was reduced from 55 gm. to 31 gm. per week and actual positive balances established. This animal refused some feed during the period from April 1 to 8, but up to that time its appetite had been keen and vigorous.

The record of Animal 3, Table III, is in complete harmony with the record of the other two animals. With the non-saponifiable material fed in capsules, the fecal calcium output per week was 55 to 60 gm.; but when this material was dissolved in corn oil and administered, the fecal calcium dropped to an output of from 30 to 35 gm. per week and positive balances were established.

It seems to us that these records are convincing proof that the antirachitic factor of cod liver oil is as effective an agent in promoting calcium assimilation in a lactating animal as it is in a growing one. The data also show that for effective results with this type of

TABLE IV.

Record of Calcium Balance and Milk Production of Animal 4.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Period 1. No addition.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
June 3–10	34.82	10.86	0.44	46.12	39.48	−6.64	6315
" 10–17	38.90	11.20	1.14	51.24	39.48	−11.76	5527
" 17–24	43.33	9.63	1.12	54.08	39.48	−14.60	5537
" 24–July 1	37.05	10.19	2.00	49.24	40.02	−9.22	5480
Period 2. 15 cc. of refined corn oil added daily.							
July 1–17	45.87	11.15	1.69	58.71	43.26	−15.45	5605
" 7–14	47.85	11.75	1.40	61.00	43.26	−17.74	5625
" 14–21	42.14	11.13	1.29	54.56	46.02	−8.54	5205
" 21–28	49.29	10.42	1.54	61.88	52.92	−8.96	4740

animal, the antirachitic vitamin must be dissolved in some suitable solvent.

To furnish definite proof that the corn oil used as a solvent for the non-saponifiable material was not an important factor in improving the calcium assimilation in these animals, an experiment was run in which after a preliminary feeding of the basal ration 15 cc. of corn oil were fed daily. The record of these results is shown in Table IV and makes it very evident that the corn oil itself had no influence on calcium assimilation. The fecal calcium was not reduced by its feeding and negative calcium balances remained quite as large as in the preliminary period.

As evidence that after adjustment to *ordinary cod liver oil feeding* the milking goat will show improved calcium assimilation, the following experiment was performed. On February 25, 1926, two dry goats, due to freshen the latter part of March, were placed on our ration of 1 part of chopped alfalfa to 2 parts of a grain mixture composed of 59 parts of yellow corn, 25 of wheat bran, 15 of oil meal, and 1 of common salt. To this ration was added 2 per cent of medicinal cod liver oil. To secure this proportion 3 pounds of cod liver oil were mixed with 97 pounds of the grain mixture. The animals were allowed what they would consume

TABLE V.

Record of Calcium Balance and Milk Production of Animal 5.

Period.	CaO in feces.	CaO in milk.	CaO in urine.	Total CaO excreted.	Total CaO intake.	Balance per week.	Milk per week.
Period 1. Ration contained 2 per cent of medicinal cod liver oil.							
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Apr. 8–15	27.39	18.47	1.14	47.00	38.67	−8.33	8105
" 15–22	35.10	15.10	0.82	51.02	38.67	−12.35	6805
" 22–29	35.15	13.65	0.43	49.23	38.67	−10.56	6440
Period 2. Ration contained 2 per cent of medicinal cod liver oil plus an addition of 14 gm. of CaO as calcium carbonate per week.							
Apr. 29–May 6	43.62	12.01	0.53	56.17	52.70	−3.47	5980
May 6–13	44.01	10.33	1.03	55.37	52.67	−2.70	5439
" 13–20	42.75	9.12	0.54	52.41	52.67	+0.26	5014
" 20–27	42.88	8.89	1.07	52.84	52.67	−0.17	4585
" 27–June 3	41.64	8.94	1.08	51.66	52.67	+1.01	4660
June 3–10	45.11	8.16	0.92	54.19	52.32	−1.87	4745

Cod liver oil feeding begun 6 weeks before cage record was taken.

of this mixture, which contained 0.73 per cent of CaO. It was planned to accustom the animals to the ration containing the cod liver oil and then, after they had freshened and were well established in their lactation, to transfer them to our metabolism cages for a calcium balance. One animal freshened in late March and the other in early April. The one freshening in April was a young animal (first gestation period) and was greatly disturbed by the milking process; consequently it was not used for experimental purposes. The other animal was placed in the matabolism cage

April 8 for a balance record and showed a negative calcium balance over a 3 weeks period. It was quiet and its appetite and consumption were good throughout the entire period which lasted 9 weeks. The fecal calcium was very low which indicated that very good calcium absorption was taking place. This animal was a liberal milker, giving 6 to 8 liters per week. It occurred to us that it was entirely possible that the calcium intake was too low in amount although one-third of the ration was alfalfa hay. Consequently we increased the calcium intake by feeding 14 gm. of CaO per week as calcium carbonate, giving us a ration with 1.00 per cent of CaO. The calcium carbonate was mixed in the grain. As seen in Table V, where the record of this animal is shown, the fecal calcium increased somewhat after the administration of the extra calcium, but a considerable proportion was utilized in decreasing the negative balance. In fact, this animal was practically in equilibrium on this somewhat higher level of calcium intake for a period of 6 weeks in the metabolism cage. We think more data on this particular phase of the problem should be secured but the deductions from the record of this animal point strongly to the view that when accustomed to cod liver oil feeding the lactating goat will show improved rather than impaired calcium assimilation.

SUMMARY.

1. The non-saponifiable fraction of cod liver oil fed to lactating goats in capsules as a solid and pasty mass was not effective in improving calcium assimilation. When, however, this non-saponifiable material was redissolved in corn oil and then administered, marked improvement in calcium assimilation resulted.

2. The corn oil itself had no effect upon calcium assimilation.

3. Accustoming the animal to direct cod liver oil feeding through an extended preliminary period before actual metabolism work was undertaken showed that this material was effective in improving calcium assimilation in the lactating animal. These results also support the view that the rôle of vitamin D in the lactating animal is comparable to its rôle in the growing animal.

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CHANGES IN THE INORGANIC CONSTITUENTS OF BLOOD IN SUPRARENALECTOMIZED CATS AND RABBITS.*

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As a part of our study of suprarenal function, the inorganic constituents of the blood and their alteration during varying degrees of suprarenal activity have been investigated with the results reported here. Part I deals with the blood changes that accompany the marked and rapidly progressing suprarenal deficiency of cats. Analyses were made on both the whole blood and plasma of normal and suprarenalectomized cats, taken, in the latter case, usually shortly before death was expected. Part II deals with the progressive but less severe grades of suprarenal deficiency of rabbits.

We have found in this laboratory *macroscopic* accessory suprarenals in about 90 per cent of suprarenalectomized rabbits but in only 6 per cent of our cats.[1] This accounts at least in part for the more gradual onset of symptoms and longer survival of rabbits. Blood obtained from an ear vein of the animals was analyzed several times before and after suprarenalectomy. Naturally not enough blood could be obtained to make a complete analysis; only the sodium and potassium content of the plasma was determined, since the more significant changes seemed to occur in these elements.

Part I.

Influence of Suprarenalectomy on Na, K, Ca, Mg, Cl, Inorganic P, and Total P Content of Blood and Plasma of Cats.

Experimental Procedure.—Control cats were anesthetized with ether and quickly exsanguinated through a cannula inserted into

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[1] Unpublished data to be reported later.

a carotid artery. This took not more than 15 or 20 minutes from the time anesthesia was commenced. The blood was collected in bottles containing twice recrystallized lithium citrate, 3 mg. per cc. of blood. The specimens were combined, and part was at once centrifuged for 40 minutes at 2000 revolutions per minute in calibrated 15 cc. centrifuge tubes covered with a rubber cap to prevent evaporation; the plasma pipetted off and analyzed. Another analysis of whole blood was made. From these two series of determinations and from the percentage of red blood cells in whole blood, the amount of the inorganic constituents of the erythrocytes could be roughly calculated. Such analyses were made on the blood of eleven cats and were taken as "normal" values with the methods used.

The suprarenalectomies were performed by Dr. David Marine in the following way: Under ether anesthesia a dorsal skin incision about 8 to 10 cm. long was made over and parallel to the back bone. Lateral incisions were then made through the muscles below the last rib and in general parallel to the ribs. The glands were mobilized, ligated, and removed. The operation on the right side is entirely extraperitoneal, while on the left a small opening into the peritoneum usually occurs in separating the peritoneum from the ventral surface of the gland. The time from beginning to end of anesthesia varied from 30 to 40 minutes. Shaving and cleansing the operative field took up about half of this time. We are also indebted to Dr. Marine for the postmortem and histological examinations.

Special efforts were made to keep the animals warm and clean after the operation. Frequent inspections were made daily. The diet was varied and made attractive. Kidney, liver, spleen, heart, other muscle, salmon, milk, and bread were used.

Twelve to 24 hours before death was expected the suprarenalectomized cats were bled from the carotid in the same way that the controls were. With sufficient experience one can usually predict death within 24 hours. Cat 261, however, was sacrificed 3 days after suprarenalectomy when still in good condition. It would undoubtedly have lived several days longer. Cats 259 and 271, that had had only one suprarenal removed, were also in good condition when sacrificed.

Method of Analysis.—Chlorides were determined by the method

of Van Slyke (1). For water a 1 cc. sample was weighed and dried to constant weight over P_2O_5 in a vacuum desiccator at a pressure of about 15 mm. The other determinations were made on the filtrates of blood and plasma after deproteinizing with trichloroacetic acid. Inorganic phosphate was determined by the method of Bell and Doisy (2), total acid-soluble P by our modification of the same method (3). Na, K, and Ca estimations were made by the Kramer and Tisdall (4) methods with the slight changes noted below, and Mg by the Briggs (5) method. Sodium: The protein-free filtrate was evaporated to dryness with 4 to 5 cc. of concentrated nitric acid. We could purchase no satisfactory potassium pyroantimonate. This reagent was prepared according to Schou (6), after which no difficulty was encountered. Potassium: After the cobalti-nitrite was added to the sample, it was found necessary to let it stand overnight for complete precipitation. To prevent creeping and subsequent loss of precipitate in the washing of the potassium cobalti-nitrite, we used water which had been shaken with distilled caprylic alcohol (as originally proposed by McLean and Van Slyke in Cl estimation). Calcium: The best conditions for complete precipitation were obtained by adjusting the reaction with dilute ammonium hydroxide, Congo red as an indicator, and allowing the solution to stand overnight. Sodium acetate was not used. Magnesium: It was necessary to use distilled ammonia which was stored in a paraffined bottle.

In all operations where samples were measured, calibrated pipettes were used. Blanks were made on all reagents. Each method was tested out on inorganic salt solutions. Salts added to blood were satisfactorily recovered. In our hands the accuracy of these methods was usually better than 5 per cent. Mg determinations were the least satisfactory. Whenever enough blood was obtained (and this was usually the case) duplicate determinations were made. In a few instances, however, owing to the asthenia of suprarenalectomized animals this was not possible.

While we believe these data are correct within the limits claimed for them by their authors, that is better than 5 per cent, we do not care to stress the question of the absolute accuracy of our figures. For the present purpose, methods which would yield comparable results were desired and with the protein-free filtrate used, they were secured. The normal and suprarenalectomized cat bloods were analyzed simultaneously.

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TABLE I.

Inorganic Constituents of Plasma of Normal Cats.

Cat No. and sex.	Approximate age.	Plasma.	H_2O	Na	K	Ca	Mg	Cl	Inorganic P.	Total acid-soluble P.	Remarks.
		per cent	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
247A M.	Adult.	71.4	91.0	381	23.0	10.5	2.4	407	6.4	6.8	
247B "	"	67.9	92.4	377	15.9	9.4	2.5	415	8.1	8.0	
252A "	"	56.0	91.7		24.4	10.1	2.4	402			
262A "	7 mos.	64.2	91.5	400	17.5	9.6	2.5	403	5.5		Blood stood 3 hrs. before-centrifuging. Distemper.
1–4 "	Grown kitten.	67.3	91.2	389		10.4	2.0	412	7.8	7.9	
265B "	Old adult.	65.4	90.9	368	19.2	9.9	2.3	427	5.9	6.4	
267B "	Adult.	59.5	91.7	379	20.6	11.1	2.4	408	6.1	6.9	
274A F.	Grown kitten.	63.0	92.1	360	17.1	11.5	1.9	422	5.1	5.8	
274B "	Young adult.	63.3	91.1	377	26.3?	9.5	1.9	428	4.9	5.4	
274C M.	" "	67.3	91.7	365	16.0		2.3	428	4.0	4.7	
280A "	Old adult.	65.1	89.5	356	17.3	10.4	2.3	409	4.1		
Average.....		64.6	91.3	375	19.0	10.2	2.4	415	5.8	6.5	

Results.—The observations on the plasmas of the normal cats are summarized in Table I, those on whole blood of the same animals in Table II. The maximum, minimum, and average water percentages of plasma are 92.4, 89.5, and 91.3 respectively. Only the plasmas of very old cats contained less than 91 per cent of water, a finding in accord with similar ones in the literature. The percentage of plasma ranged from 56 to 71.4 with an average of 64.6.

TABLE II.

Inorganic Constituents of Blood of Normal Cats.

Cat No.	H_2O	Na	K	Ca	Mg	Cl	Inorganic P.	Total acid-soluble P.	Remarks.
	per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
247A	83.2	348	28.8	8.1	3.9	368	6.7	13.1	
247B	83.3	348		7.4	3.7	375	8.1	13.1	
252A	79.2	374	27.5	7.1	3.2	355			
262A	79.2	360		7.0	2.8	358	8.1	13.1	Distemper.
1–4	82.7	359		8.2	3.6	366	8.4	16.3	
265B	81.6	360	22.1	7.2	3.2	372	6.1	12.5	
267B	79.7	358	22.5	7.5	3.2	349	6.5	14.1	
274A	81.1	356	21.3	9.9	3.9	364	5.8	11.3	
274B	80.7	368	28.3	7.7	2.9	379	5.4	10.5	
274C	81.4		19.7	7.6	3.0	376	4.8	10.4	
280A	80.2	364	22.8	7.6	3.4	375	5.6	17.0	
Average....	81.1	360	24.1	7.8	3.3	367	6.6	13.1	

The sodium content varied from 356 to 400 mg. per 100 cc. with an average of 375; potassium from 15.9 to 24.4 with an average of 19.0; calcium from 9.4 to 11.5 with an average of 10.2; magnesium from 1.9 to 2.5 with an average of 2.4; chlorine from 402 to 428 with an average of 415; inorganic phosphorus from 4.1 to 8.1 with an average of 5.8, and the total acid-soluble phosphorus from 4.7 to 8.0 with an average of 6.5. In general, the figures agree with those found by other investigators except those for sodium. The only sodium determinations on cat blood and plasma that we have found were those of Abderhalden (7) on the pooled blood of three cats and his value is somewhat lower than that

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TABLE III.

Cat No. and sex.	Approximate age.	Duration of life after suprarenalectomy.	Plasma.	H_2O	Na	K	Ca	Mg	Cl	Inorganic P.	Total acid-soluble P.	Remarks.
Inorganic constituents of plasma of unilaterally suprarenalectomized cats.												
		days	*per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
259 M.	Old adult.		68.6	90.2	371	13.3	10.9	1.6	418			Sacrificed after 10 days.
271 "	Adult.		65.8	90.2	353	14.2	10.6	1.8	418	3.8	4.2	" " 21 " draining infected sinus.
Inorganic constituents of plasma of bilaterally suprarenalectomized cats.												
261 M.	Adult.	3	58.9	90.7	336		9.6	2.3	386	6.2	7.0	Eating; in good condition when sacrificed.
269 F.	"	7	63.5	89.8	344	21.5	13.3	3.2	391	7.1	6.8	
274 "	"	3	53.0	90.2	294	28.0	10.1	2.9	422	5.9	5.9	
273 M.	Young adult.	7	52.3	90.7	338	31.0	11.0	4.0	392			
267 "	" "	22	67.8	91.9	292	20.6	10.0	2.8	358	4.8	6.1	Semicomatose when sacrificed.
281 F.	Adult.	3	57.6	89.0	348	32.0	10.7	3.2		6.0		Partial thyroidectomy.
279 M.	"	5	41.6	91.0	297	43.1	14.5	4.0				
283 F.	"	6	62.6	90.0	307	26.8	9.7	4.1		7.2		A 2 mm. accessory suprarenal found. Partial thyroidectomy.

												found. Partial thyroidectomy.
282 M.	"	6	60.2	89.0	324	24.2	10.5	2.5	389	6.3	6.7	Partial thyroidectomy.
287 F.	Young adult.	2	46.7	89.4	299	28.7	11.2	2.7				
288 M.	Old adult.	6	58.2	89.1	328	26.7	10.8	3.9	363	6.5	6.4	
286 F.	" "	7	64.3	89.5	327	24.9	16.3	2.8	325	7.0	6.8	
Average.....			57.2	90.0	320	28.0	11.5	3.2	378	6.3	6.5	

Inorganic constituents of plasma of bilaterally suprarenalectomized cats influenced by complicating factors.

268 M.	Adult.	2	62.1	90.5	363	21.3	10.3	2.1	382	7.3	7.9	Trauma at operation.
272 "	Old adult.	12	63.7	88.8	350	60.0	13.8	4.2	393	9.9	10.6	Terminal bronchopneumonia.

found by us with the Kramer and Tisdall method. From the whole blood and plasma results, and from the approximate percentage of plasma in whole blood, the content of the various ions in the erythrocytes is calculated. The amount of calcium in the erythrocytes is thus found to be about 2.0 mg. per 100 cc.

TABLE IV.

Cat No. and sex.	H_2O	Na	K	Ca	Mg	Cl	Inorganic P.	Total acid-soluble P.
Inorganic constituents of blood of unilaterally suprarenalectomized cats.								
	per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
259 M.	81.8	365	25.1	9.0	2.8	374	9.8	12.6
271 "	81.6	349	27.0	8.9	3.6	365	4.9	10.5
Inorganic constituents of blood of bilaterally suprarenalectomized cats.								
261 M.	79.6	320		6.8	4.1	345	7.3	13.0
260 "	79.3	314	34.1	6.9	4.7	346		
269 F.		308				340		
274 "	76.8	256	36.4	6.6	4.5	351	6.8	12.4
273 M.	78.3	326	36.8	7.1	4.3	332	8.6	
267 "	83.1	306	30.2	7.6	4.2	313	6.5	12.5
282 "	79.2	324	30.8	7.1	4.2	347	7.0	13.0
288 "	78.1	303	42.3	7.5	4.1	321	7.6	13.9
286 F.	80.0	329	33.2	11.6	4.1	335	7.4	13.4
Average..	79.3	310	34.8	7.7	4.3	337	7.3	13.0
Inorganic constituents of blood of bilaterally suprarenalectomized cats influenced by complicating factors.								
268 M.	80.5	336	32.9	7.6	3.2	435	8.0	13.7
272 "	80.0	345	50.4	10.1	4.1	365	10.3	15.7

However, the separation of cells and corpuscles by centrifuging at 2000 revolutions per minute is by no means sharp, so that 2.0 mg. per cent is too high a value for the calcium content of the red blood cells. They contain probably less than 1 mg. per cent and perhaps no calcium at all, as Kramer and Tisdall (8) and earlier workers have found in the case of man. These investigators

have likewise found practically no sodium in human erythrocytes. In the case of the cat, however, we must conclude from our data that sodium is present, a finding noted previously by Abderhalden.

The analyses of the plasma of two unilaterally suprarenalectomized cats and of twelve bilaterally suprarenalectomized cats are presented in Table III. In Table IV will be found the results of analyses of blood of two unilaterally and nine bilaterally suprarenalectomized cats. In addition, the results on two other suprarenalectomized cats which were affected by complicating factors are appended at the bottom of the tables. One of the animals, Cat 272, at autopsy was found to have a terminal bronchopneumonia. The other cat, No. 268, was badly traumatized during the operation. The left gland was firmly attached and in such a way that we were unable to secure a good exposure. The operation was prolonged unduly, during which time the very important and sensitive surrounding tissues were badly injured. At autopsy considerable hemorrhage and other evidences of trauma were noted. Some of the results are quite out of line with those on the other suprarenalectomized cats and they are therefore not included in the averages. These data would ordinarily not be reported but they show how completely the effects of suprarenalectomy may be masked by poor surgery.[2]

From Tables III and IV it will be seen that there are definite changes in the salts of blood and plasma of suprarenalectomized animals. Plasma averages only 57 per cent of the total blood against 65 per cent normally. In one suprarenalectomized cat it was only 42 per cent, in others the plasma percentage was within

[2] Unfortunately, in the greater part of the literature of physiology in which suprarenalectomy is one of the procedures used, the influence of trauma and bad after care has far outweighed the influence of suprarenalectomy. This point has been stressed recently by Stewart (9); it cannot be over emphasized. That most investigators who have used suprarenalectomized cats, dogs, or rabbits have been studying the effects of trauma and shock rather than those of suprarenalectomy is evident from the fact that even when the suprarenals have been removed in two stages, their animals have usually died in less than 48 hours after removal of the second gland. Dogs and cats will survive a clean suprarenalectomy on the average 5 or 6 days, while most rabbits, because of the more frequent occurrence of accessories, will usually survive double suprarenalectomy at least several weeks and often indefinitely.

the normal range. The plasma solids are uniformly increased on the average from 8.7 to 10 per cent, which indicates a plasma concentration effect of 15 per cent. Lucas (10) and Stewart and Rogoff (11) found similar changes in dogs. Yet, in spite of this concentration, we find sodium and chlorine decreased in the plasma of all the suprarenalectomized cats, amounting on the average to 15 per cent for sodium and 9 per cent for chlorides. Similar decreases in blood chlorides have been observed by Lucas in suprarenalectomized dogs.

Potassium and magnesium increase 9 and 0.8 mg. respectively on the average, which increases are 42 and 23 per cent above normal and well beyond any effect that could be ascribed to concentration. Kisch (12) reports that the serum calcium of two suprarenalectomized rabbits first decreased and later rose above the normal. We find little change in the plasma calcium of most of the suprarenalectomized cats, which is what Lucas also found in the dog. In three of our cats, however, there are definitely increased values; namely, in Cats 269, 279, and 286. The analyses were all made in duplicate and there is no reason to question their accuracy. Except for these cases, we have never encountered such figures in the analysis of many animal and human plasmas. The possible significance of these high figures will be discussed later. No noteworthy changes were observed in the inorganic or total acid-soluble phosphorus. These results differ from those of Lucas who found a progressive rise in the blood phosphates of suprarenalectomized dogs.

The changes found in blood are of about the same magnitude as those found in plasma. The approximate values of the inorganic ions for blood corpuscles were calculated and found to show the same variations that these elements show in plasma, but not to the same extent. The average decrease in plasma sodium is 15 per cent, for corpuscles 10 per cent; the average potassium increase in suprarenalectomized cat plasma is 42 per cent, for corpuscles 36 per cent; the average magnesium increase in plasma is 23 per cent, for corpuscles 11 per cent, etc. The ions of the erythrocytes seem to undergo a secondary change as a result of being bathed in plasma the composition of which is changing.

In the unilaterally suprarenalectomized cats, we find a slight increase in total solids, practically no change in most of the ions

except for a possible decrease in the plasma potassium. In both cats the potassium level is below the lowest normal value and about 25 per cent below the average normal. We have too few data to do more than point this out.

A few illustrative protocols are given.

Cat 267.—Male, black; young adult. Mar. 9, 1926. Weight 3168 gm. Clean and fat. Under ether removed right and left suprarenals, also tiny accessory suprarenal on left; 9.00 to 9.40 a.m. Mar. 10. Drinks all milk. Mar. 11. Eats milk and little fish; slight discharge from eyes; condition fair. Mar. 13 to 25. Eats well; bright; occasionally shows weakness in hind legs. Mar. 28. Eats well; weight 2893 gm. Mar. 29. Slight nasal discharge; eats meat. Beginning to decline. Mar. 30. Eats little meat; sleeps most of time. Mar. 31. Eats about 50 gm. of meat. Declining fast. At 4.30 p.m. semicomatose. Sacrificed to get blood.

Necropsy.—Lymph glands, prominent. Thyroid, small, withered. Thymus, large, cellular. Lungs, free from consolidation. Heart, small, firmly contracted. Stomach, numerous tiny erosions having small blood clots at sites. Liver, marked nutmeg appearance, soft, friable. Spleen, somewhat enlarged, soft, dark red. Pancreas, slightly hyperemic. No evidence of suprarenal tissue found at sites. Sutures visible, perfectly healed. Peritoneal cavity, clean. Kidney, right, some extravasation of blood beneath capsule, result of operation; cortex thickened, brownish yellow and soft. Testes, small, undeveloped, not mature. Abundant fat in all depositories. Back wounds, clean, healed.

Cat 261.—Male, dark tiger; adult. Jan. 27, 1926. Weight 3301 gm. Clean and well nourished. Under ether removed right and left suprarenals complete; 11.35 a.m. to 12.05 p.m. Very slight hemorrhage on left. Jan. 28. Active but not eating. Jan. 29. Eats little food; dull. Jan. 30. Eats meat; weak, condition fairly good. Would undoubtedly have lived several days longer. Sacrificed to obtain blood before terminal effects were present.

Necropsy.—Weight 3225 gm. Lymph glands, normal. Thyroid, normal. Thymus, present, involuted. Lungs, free from consolidation. Heart, in systole, right ventricle dilated. Stomach, empty, one tiny ulcer. Liver, brown. Spleen, normal. Pancreas, pale. Suprarenal sites, clean. Testes, normal. Back wound, somewhat moist, no pus.

Cat 269.—Female, three colored; adult. Mar. 9, 1926. Weight 3180 gm. Clean, well nourished. Under ether removed right and left suprarenals; 11.30 a.m. to 12.10 p.m. In advanced pregnancy. Mar. 10. Drinks milk. Mar. 11. Aborted; three young born this morning, one alive. Removed young which were about 2 weeks from term. Eats 50 gm. of meat and salmon. Mar. 12. Active and hearty. Eats about 200 gm. of meat and salmon. Mar. 13 to 14. Continues active and well. Mar. 15. Growing dull, sleeps a lot; eats 25 cc. of milk and 50 gm. of meat. Mar. 16. Eats 25 gm. of meat and little milk. Walks about actively. 4 p.m., labored respiration, refuses food for first time since operation. Sacrificed.

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Necropsy.—Weight 2815 gm. Lymph glands, normal. Thyroid, small, translucent. Thymus, present, cellular. Heart, normal. Stomach, empty, no ulcers, mucosa pale. Duodenum, numerous very hyperemic spots. Liver, normal. Spleen, small. Pancreas, pale. Suprarenal sites, left, clean; right, considerable hemorrhage at site; no definite suprarenal tissue found on dissection through small encapsulated blood clot. Kidneys, small, cortex brownish yellow, pale. Ovaries, no corpora lutea visible on left; two atrophic ones on right. Fat, yellowish, considerable fat in all depositories. Back wound, clean.

DISCUSSION.

In considering these observations, there are certain errors that may result from the way the blood was obtained. We assumed that the effect of the anesthetic would be small since the period of narcosis was short, 10 minutes or less, and, in any case, the effect would be the same in both normal and suprarenalectomized cats. Anesthesia was stopped as soon as the carotid had been cannulated and bleeding commenced. In order to get sufficient blood for analysis, especially from small animals, it was necessary to exsanguinate them nearly completely, a matter of 3 to 5 minutes. During this time the tissues would probably give up some tissue fluid to the circulating fluid and so might dilute it. We cannot say how great this effect may be. It would probably not be as great in suprarenalectomized animals as in normals because it is not possible to obtain as much blood from the former.[3]

Sodium represents 4.3 per cent of the total solids of our normal cat plasma but only 3.2 per cent of the solids of suprarenalectomized cat plasma, a decrease on this basis of 25 per cent. This we believe is the most important observation of our experiments. There is a concomitant but smaller decrease in blood chlorides. In terms of milli-equivalents, the amount of chlorides decreases 1.1 units, while the amount of Na plus K decreases 2.2 units, despite an increase of 0.2 milli-equivalents in potassium. This leads us to infer that suprarenalectomy influences sodium metabo-

[3] Lucas and others speak of the difficulty of obtaining blood from suprarenalectomized animals and they have ascribed this to the thickening of the blood. While it is true that the blood is more viscous than in normal animals, we are inclined to believe that the main reason for this difficulty is not blood concentration but the cardiovascular asthenia that is one of the most characteristic symptoms of suprarenal deficiency.

lism more than that of chlorine, and that the latter follows the former.

Leaving out of consideration the CO_2 content of plasma, which could not be determined because of experimental limitations, the average excess of cations over anions is 5.7 milli-equivalents in the normal cats, while in the suprarenalectomized animals this figure is 4.4 milli-equivalents. If there were no corresponding changes in the plasma CO_2, this would speak for a shift toward an acidosis. Certainly in these data we see no evidence of an alkalosis in the suprarenalectomized animals, a supposition which has been advanced by Bornstein, Holm, and Gremels (13, 14) and by Keitel (15).

The sum of the anions and cations of normal cat plasma in these experiments varies from 28.2 to 29.9 milli-equivalents, the average being 29.2. Corresponding figures for the suprarenalectomized cats are 25.2 to 27.5 and 26.3. That is, there is a definite decrease in the total salts of the plasma, despite an increase in some ions, a finding that is in agreement with Stewart and Rogoff's (11) observation that suprarenalectomy causes a decrease in the conductivity of blood and serum, but in conflict with the observations of Keitel and of Rohdenberg and Krehbiel (16). Keitel found increases in the serum sodium, potassium, and calcium of suprarenalectomized dogs. Rohdenberg and Krehbiel found much greater increases in the sodium and calcium of suprarenalectomized rat blood and a decrease in potassium. The total salts increased 400 to 800 mg. per 100 cc. While the response to suprarenalectomy of dogs and rats may differ from that of cats, we are inclined to suspect that these observations were made on animals *that were suffering from the effects of trauma, bad after care, or both*, rather than from the effects of the suprarenalectomy. No autopsy protocols are given in either of these papers so it is difficult to form a definite judgment on this point. How trauma may obscure the effects of suprarenalectomy has been discussed; the reader is again referred to the results on Cat 268, Tables III and IV.

In the blood corpuscles the sum of the cations and anions calculated from whole blood and plasma analyses show little changes from the normal.

The ratios of the various cations to one another have been shown to be of the greatest importance in the maintenance of various cell

functions. Although there have been many studies of this question the exact part played by each of the ions in various processes is still in dispute. But it can be definitely stated that alteration of these relations causes profound metabolic disturbances. The analyses we have made show that the ratios of sodium to potassium, magnesium, etc., in plasma and blood undergo even greater changes than the absolute values because, while there is a decrease in some of the ions, others increase. For example, the average plasma sodium to potassium ratio for normal cats is found to be 20.0; for suprarenalectomized cats it is 11.8, a drop of 41 per cent. Similarly there is a fall in the sodium to magnesium ratio of 39 per cent.

The relation of plasma potassium to calcium is supposed by many investigators to have special significance. In the normal cats, this ratio is 1.86, in unilaterally suprarenalectomized cats 1.28, and in bilaterally suprarenalectomized cats 2.43; that is, a *decrease* of 31 per cent in the two unilaterally suprarenalectomized animals and an equally great *increase* where double suprarenalectomy was done. This reversal could be looked on as a compensatory effort after partial suprarenalectomy and may be a good measure of the degree of suprarenal deficiency.

The highest calcium content of normal cat (adult) plasma that we have observed is 11.5 mg. per 100 cc.; the average of ten animals is 10.2. Most of the analyses of cat plasma of suprarenalectomized animals fall within the normal range, but three of the twelve had the unusually high plasma calcium levels of 13.3, 14.5, and 16.2 mg. per 100 cc. respectively. These cats were all adults. As has already been noted, there is no reason to suspect the accuracy of the figures.

An explanation of these unusually high figures can be offered on the basis of a concept developed in this laboratory during the past 6 years of study of the suprarenal. This hypothesis states that the suprarenal gland normally exerts an inhibitory or regulatory influence on many activities of the mammalian body. By removing this regulatory influence (by suprarenal extirpation) the rate at which these functions go on should be augmented, if this assumption be correct. Marine and Baumann (17, 18) have shown that a severe but sublethal suprarenal insufficiency causes a higher metabolism and that this in turn is dependent upon increased thy-

roid activity. Those familiar with the pathologic findings of suprarenalectomized animals recognize that there is reason for believing that the pancreas, stomach, thymus, and other lymphoid tissues, as well as other organs, are functioning at greatly increased rates. However, these pathological changes are not *all* seen in *all* suprarenalectomized animals. While the findings are highly variable, they occur frequently enough to show that they result from removal of the suprarenal glands. It may well be that the parathyroids are also stirred to greater activity by suprarenalectomy and that the pouring out of more than the usual amount of parathyroid secretion causes the very high calcium values that have been found.

In several investigations in which suprarenal injury is known to play a part, observations similar to ours have been made. In severe burns the suprarenal is badly injured; in fact, perhaps the most grave symptoms are caused by the secondary effect on these glands. Davidson (19) and Underhill (20) have found a fall of 20 per cent or more of blood chlorides in cases of cutaneous burns. We believe if the sodium content of this blood had been determined an equally great fall in sodium would also have been noted. And perhaps the beneficial effect of forcing fluids in cases of burns, noted first by Underhill (20), is caused by the sodium chloride in the fluid that is administered, the water being only of secondary importance.

One of the most prominent findings in animals dying in anaphylactic shock is injury of the suprarenals. Azzi (21) believes there is a diminution in the cations of guinea pig blood in anaphylactic shock, while Zunz and La Barre (22, 23) noted a decrease in both the sodium and chlorine in guinea pig serum.

Finally, one additional bit of evidence that a lowering of the sodium and chlorine content of blood is one of the most important derangements occurring in suprarenalectomized animals is suggested by experiments that were made with Dr. Marine several years ago and that are being submitted for publication separately. While attempting to prolong the lives of suprarenalectomized cats with various suprarenal extracts, we had reason to believe that some seemingly potent extracts owed their effect to the salt in them. This led to our injecting various salts into suprarenal-

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ectomized animals and practically all the sodium salts used prolonged life. Stewart and Rogoff (24) were able to prolong the lives of suprarenalectomized dogs by injection of Ringer's solution. We believe this result was chiefly due to the sodium chloride of the Ringer solution.

For the present the significance of these observations needs no further discussion. Whether some or all of the changes we have noted are only prodromal effects or are those of suprarenal deficiency will have to be decided by further study.

Part II.

Influence of Suprarenalectomy on Sodium and Potassium Content of Rabbit Plasma.

Experimental Procedure.—Eight rabbits were used. They had had one suprarenal removed for some other work before being used for these experiments. Two of the eight, Rabbits 591 and 592, had also had their thyroids removed. At autopsy these were found to have large fragments of regenerated hyperplastic thyroid tissue so that they were suffering from only partial thyroidectomy. About 12 to 14 cc. of blood were obtained from a marginal ear vein and clotting was prevented by hirudin. The blood was at once centrifuged for 40 minutes at 2000 revolutions per minute, plasma readings taken, and samples of plasma measured out for water, sodium, and potassium determinations, using the same methods described in Part I. After two or three specimens from the rabbits had been analyzed over a period of 3 weeks, the second suprarenals were removed and bloods analyzed, usually at weekly intervals.

The rabbits were fed on our stock ration of alfalfa hay and oats, and, in addition, had some greens or carrots twice weekly. After the second suprarenal had been removed, the amount of the ration was increased to prevent a loss of weight that usually occurs from the higher metabolism of suprarenalectomized rabbits.

Results.—The results are shown in Table V. A few illustrative protocols are also given.

Rabbit 591.—Male; light gray; born Mar. 16, 1925.

May 9, 1925. Under ether removed most of right and left thyroid lobes. Weight 722 gm. Dec. 17. Weight 2142 gm. Under ether removed left

suprarenal completely. Considerable hemorrhage. Jan. 1, 1926. Wound healed. Used in connection with other experiments until Apr. 27, during which time took few small samples of blood. Apr. 27. Took 12 to 14 cc. blood weekly. June 2. Under ether completely removed right suprarenal, also a 1.5 mm. accessory suprarenal to left of inferior cava. Time, 11.55 a.m. to 12.15 p.m. June 3. Active. Weight 1885 gm. June 7.

TABLE V.

Influence of Suprarenalectomy on Na and K of Rabbit Plasma.

Date.	Plasma.	H_2O	Na	K	Plasma.	H_2O	Na	K
	Rabbit 586.				Rabbit 594.			
1926	per cent	per cent	mg. per cent	mg. per cent	per cent	per cent	mg. per cent	mg. per cent
May 7	53.3		383	20.5	58.0	91.5	380	20.8
" 13	57.0	91.8	359	20.4	56.2	91.5	367	20.5
" 20		92.4	393	23.2		91.3	380	20.6
	Second suprarenal removed on June 2.							
June 8	50.8	91.7	311	23.2	55.9	91.0	352	20.8
" 15					53.8	92.3	323	21.6
June 14. Died.					June 17. Died.			
	Rabbit 591.				Rabbit 592.			
Apr. 27	66.3	91.4			62.2	91.7	349	22.4
May 13	67.3	91.4	363	18.5	54.1	91.4	355	20.3
" 20	66.2	91.6	371	20.6			383	22.3
	Second suprarenal removed on June 2.							
June 8	63.8	92.1	325	24.7	58.9	92.2	368	22.9
" 15	63.3	91.8	319	26.3	57.2	91.8	357	19.7
" 22	69.8	92.7	314	17.9	59.4	92.1	355	21.9
July 6	68.1	92.7	296	21.9	58.2	91.8	363	19.5
Aug. 5					56.2	91.7	312	23.1
" 12					65.5	92.7	342	23.1

June 11 to 16. Clinically in bad condition; rough coat.
June 17. Fed 50 mg. of KI.
" 25. Splendid growth of soft fur over shaved area; eats better.
July 8. Found dead.

June 15. Not eating full ration
" 17. Fed 50 mg. of KI.
" 25. Hair growing over shaved area; not as well covered as Rabbit 591.
Sept. 15. Died at 3.30 p.m.

TABLE V—*Concluded.*

Date.	Plasma.	H_2O	Na	K	Plasma.	H_2O	Na	K
	Rabbit 598.				Rabbit 599.			
1928	*per cent*	*per cent*	*mg. per cent*	*mg. per cent*	*per cent*	*per cent*	*mg. per cent*	*mg. per cent*
Apr. 29	57.0	91.8		19.6	58.6	92.3	375	20.2
May 13	55.8	92.1	362	20.6	57.3	92.2	403	19.2
" 20	57.4	91.9	374	20.6	61.3	92.5	357	21.1
	Second suprarenal removed on June 3.							
June 8	57.3	91.6	353	20.5	59.2	92.3	351	20.7
" 15	57.9	91.9	361	19.5	58.8	91.9	350	15.9
" 22	58.3	91.6	373	17.8			359	20.3
July 6	56.6	91.9	367	16.1	54.8	91.4	328	18.5
Aug. 5	59.4	91.5	312	22.7	57.5*		339	23.7
" 12					65.3		301	18.1
June 25 to July 12. In poor condition; eating little; diarrhea; coat rough. Aug. 5. Condition excellent since July 12. Sacrificed to terminate experiment.					Still alive. In excellent condition.			
	Rabbit 600.				Rabbit 601.			
Apr. 29	58.9	92.1	368		58.5	92.0	371	22.2
May 7	57.7		377	25.4				
" 13	59.1	92.4	365	18.1	59.9	92.3	365	19.2
" 20	58.4	92.1	366	21.2	59.8	91.8		20.5
	Second suprarenal removed on June 3.							
June 8	56.6	91.8	377	19.7	59.8	91.6	386	15.6
" 15	59.2	92.1	369	15.9	64.4	91.7	356	18.1
" 22	52.8	91.1	354	18.2	58.0	91.2	359	18.6
July 6	57.1	91.5	364	17.9	57.6	91.8	300	16.9
Aug. 12					63.2	92.2		18.3
July 17. Died during night.					June 7 to 9. Soft stools; eating well. Still alive. In excellent condition.			

*Approximately.

Ravenous appetite. June 11. Eats only part of ration. Weight 2010 gm. June 15. Continues to eat only part of ration; fur which had become glossy after operation again rough and dry. June 17. 25 mg. of KI by mouth. June 19. 25 mg. of KI by mouth. Weight 1820 gm.; eating more. June 25. Splendid growth of soft fur over shaved area; active and eating full ration. June 29. Eats only greens and hay, but fairly active. July 1. Eats no oats. July 8. Found dead in a.m. Had not eaten carrots of yesterday.

Necropsy.—Weight 1784 gm. Lymph glands, not prominent. Thyroid, very vascular fragments on right and left. Left = 3 × 2 mm.; right = 2 × 1.5 mm. Parathyroids, bright. Thymus, present, markedly congested, both lobes very distinct, probably regenerating. Lungs, edema and congestion of all lobes, little consolidation. Heart, small. Stomach, distended with food, considerable autolysis. Many ecchymoses in cardiac portion. Liver, grayish brown, not congested, reduced in size. Spleen, very small, pale. Pancreas, slightly congested. Suprarenal sites, clean sutures present; no accessories seen. Peritoneal cavity, clean. Kidneys, small, no pitting. Testes, appear normal. Fat, scant. Back wounds, clean and healed. Shaved area covered with thick soft fur. Major cause of death, suprarenal insufficiency, uncomplicated.

Rabbit 598.—Female; white; born Nov. 12, 1925. Dec. 17, 1925. Weight 519 gm. Under ether completely removed left suprarenal. Jan. 1, 1926. Wound healed. Used for other experiments until Apr. 27, during which time took a few small samples of blood. Apr. 27. Took 12 to 14 cc. of blood weekly. June 2. Weight 2325 gm. June 3. 9.00 to 9.25 a.m., under ether removed right suprarenal completely, slightly enlarged. June 7. Eating well. June 11. Weight 2265 gm. June 18. Weight 2305 gm. Continues to eat full ration. June 21. Very flaccid. June 25. No muscle tone. June 26. Has eaten no oats for last 4 days. June 29. Looks better; active; good muscle tone. June 30. Slight snuffles. July 12. Diarrhea and snuffles; coat rough. Aug. 4. Has been in excellent condition since last note. Aug. 5. Condition excellent. Sacrificed to terminate experiment. Weight 2408 gm.

Necropsy.—Lymph glands, not enlarged. Thyroids, normal, grayish, soft. Parathyroids, normal. Thymus, active; cellular; about normal size of full development. Lungs, normal. Heart, small, normal. Stomach, full of food. Liver, normal. Spleen, small, two tiny accessory spleens in pancreas. Pancreas, not congested. Suprarenal sites, clean. Three accessory suprarenals on peritoneum of left side; one 6 × 4 mm. on peritoneum, another on anterior surface of inferior vena cava above right renal vein, 3 × 2 mm.; a possible third accessory near upper pole of right ovary. Peritoneal cavity, clean. Kidneys, normal. Uterus, hyperemic. Fat, abundant. Back wounds healed.

The plasma potassium before removal of the second suprarenal varied between 18.1 and 25.4; the average of twenty-three observations on the eight rabbits is 20.8 mg. per 100 cc. All except three

observations lay between 19 and 23. The sodium values varied between 350 and 390 mg. per 100 cc., the average being 370.

After removal of the second suprarenal, the potassium level in several rabbits dropped, slightly but definitely, below any of the preoperative levels. Later on as suprarenal deficiency becomes more severe, the plasma sodium also falls while the potassium rises to approximately its preoperative range. The initial fall in potassium noted also in unilaterally suprarenalectomized cats may be a successful effort to effect some compensation.

This is brought out better if the ratio of sodium to potassium is examined. Before removal of the second suprarenal, this ratio varies from 15 to 21. The average of twenty-two observations on the eight rabbits is 18. After the second operation this ratio at first often rises above the highest preoperative level to 23 or more by reason of a fall in the potassium. Later on, as symptoms become more severe, the sodium drops (15 to 20 per cent) and the ratio of sodium to potassium falls distinctly below its lowest preoperative figure. This is similar to the results on the cat.

As we have pointed out in Part I, the changes occurring in animals as a result of suprarenalectomy are not *all* seen in *all* animals. These changes depend at least in part on the degree of suprarenal insufficiency and this is highly variable, especially in rabbits, because of the very frequent presence of varying amounts of accessory tissue. But despite this variability, there can be no doubt that the plasma sodium does decrease in suprarenalectomized rabbits as it does in cats.

A very crude index of the severity of this deficiency may be had from the duration of life after suprarenal extirpation. The duration of life was 12 days for Rabbit 586, 15 days for No. 594, 36 days for No. 591,46 days for No. 600, and 105 days for No. 592. Rabbit 598 was sacrificed in excellent condition 63 days after suprarenalectomy, while Rabbits 599 and 601 after 150 days were still alive.

In this laboratory, suprarenalectomized animals have usually been found to have thyroids that showed evidence of increased activity. Marine has found these glands exhausted, often hyperplastic, and low in their iodine content. Because of these observations we have often fed suprarenalectomized animals, that were declining rapidly, small doses of potassium iodide and have been

astonished at the marked improvement that often follows. Scott (25) has made the same observation on suprarenalectomized cats.

The partially thyroidectomized rabbits, Nos. 591 and 592, when they were declining clinically, were given 50 mg. of KI by mouth and very soon thereafter definite improvement was noted in the amount of food eaten, and particularly in the growth of hair. The coat which had been rough and dry (cretinoid) became smooth and sleek and there was an abundant growth of new soft fur over the shaved areas. In Rabbit 591, there was a concomitant change in the ratio of sodium to potassium. Before removal of the second suprarenal it had been 18.0 and 19.6. After the operation this ratio fell to 13.2 and 12.2. After giving KI it rose to 17.5 and several weeks later it had again fallen to 13.5. Such changes are not seen in Rabbit 592.

The water determinations show no evidence of a concentration of blood solids such as was seen in the cats. This leads us to suspect that the concentration is one of the many incidental terminal changes. Likewise with the rough method we used for determination of plasma volume, no increase in the percentage of erythrocytes is seen. It should be noted, however, that compared to cats, the rabbits were in a state of good compensation.

SUMMARY.

Suprarenalectomy causes a fall in plasma sodium and chlorides. In the cat, a rise of plasma potassium, magnesium, and occasionally of calcium has been found. The diminution of plasma sodium is probably the most important of these observations. In a few other conditions where suprarenal injury occurs it has been found that a decrease in sodium and chloride content of plasma also occurs. The relation of the cations to one another is even more markedly altered in suprarenalectomized animals than the actual amounts of the cations themselves.

There is no evidence in these experiments that points to an alkalosis as one of the consequences of suprarenalectomy.

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A USEFUL COMPOUND OF HISTIDINE.*

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Owing to the lack of compounds of histidine possessing convenient solubilities the quantitative isolation of this base from such mixtures as are present in the histidine fraction obtained from plant extracts presents a difficult problem. The dichloride has usually been employed but the strongly acid solution necessary must be filtered through asbestos or glass wool, the separation is incomplete, and recrystallization of small quantities of the, often impure, material is not easy. The mono- and di-picrates of histidine have been described (1) but do not possess convenient properties. Moreover, when prepared from somewhat impure material, sirupy mother liquors are encountered from which nothing can be brought to separate. Similar difficulties are met in dealing with picrolonates.

Efforts have therefore been made to find a compound of histidine possessing a convenient solubility and a capacity for crystallization sufficient to prevent the formation of sirupy mother liquors. In view of Kossel's remarkable success with 2,4-dinitro-1-naphthol-7-sulfonic acid as a reagent for arginine (2, 3), it was natural to investigate the compounds which this substance forms with histidine.

Kossel and Gross (2) prepared the salts of a number of bases with dinitronaphtholsulfonic acid and recrystallized them from water. The analytical figures they obtained from the histidine preparation indicate that it consisted mainly, at least, of the mono-salt. Their solubility determinations suggest that this

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

may be readily separated in large, if not quantitative, yield, and moreover, since it is relatively insoluble in dilute acid, it appears likely that the histidine compound might be precipitated directly from a solution containing excess of mineral acid. This is a great advantage in practical work.

In order to test these points, 1 gm. of histidine dichloride was dissolved in 20 cc. of 5 per cent sulfuric acid and 4 gm. (a large excess) of dinitronaphtholsulfonic acid dissolved in about 20 cc. of warm water were added. Crystallization began promptly, and, after standing 48 hours, the pale yellow substance was filtered off. It was recrystallized from water, washed with a little absolute alcohol, and air-dried. The recrystallized material on heating became orange below 100°C., and decomposed in the vicinity of 224°, the same temperature as that given by Kossel and Gross. It lost approximately 7 per cent of water of crystallization on drying at 105°C., and the anhydrous substance contained 13.0 per cent of nitrogen. The theoretical nitrogen content of the anhydrous mono-salt, $C_6H_9N_3O_2 \cdot C_{10}H_6N_2SO_8$, is 14.92 per cent and of the di-salt, $C_6H_9N_3O_2 \cdot 2C_{10}H_6N_2SO_8$, is 12.51 per cent. It was evident therefore that the preparation was a mixture and that recrystallization of this compound from water in the absence of excess of reagent is inadmissible. The material was recrystallized from about 30 cc. of water containing 1 gm. of dinitronaphtholsulfonic acid, washed with a little absolute alcohol, and air-dried. It separated in pale yellow nodular aggregates of microscopic needles. When heated no color change occurred below 246°, when the substance began to darken. It decomposed with blackening and effervescence at 251–252°. The decomposition point could be raised to 254° on more rapid heating. The air-dry preparation appeared to contain one-half a molecule of water of crystallization (theory 1.13 per cent, found 1.07 and 1.14 per cent). The nitrogen, determined by the Kjeldahl method as modified to deal with nitro groups, amounted to 12.47 per cent (theory 12.51 per cent). This preparation was therefore the pure di-salt.

An attempt was made to prepare the mono-salt by treating 1 gm. of histidine dichloride dissolved in water with the theoretical amount (1.38 gm.) of dinitronaphtholsulfonic acid. The product was recrystallized from a small amount of hot water, when it separated in microscopic pale yellow needles. On heating, the

preparation lost water of crystallization below 100°, became orange in color, and finally decomposed with blackening and effervescence at 218°. It contained 6.4 per cent of water of crystallization and the anhydrous salt contained 13.75 per cent of nitrogen. It was therefore a mixture of mono- and di-salts, and it is evident that pure mono-salt cannot be obtained under these conditions.

An attempt to prepare the mono-salt from an aqueous solution containing 2 mols of histidine dichloride to 1 of dinitronaphtholsulfonic acid likewise failed. The effect of crystallization from alcoholic mother liquors was therefore tried. 2 gm. of histidine dichloride and 1.38 gm. ($\frac{1}{2}$ mol) of dinitronaphtholsulfonic acid were dissolved together in 15 cc. of hot water and 30 cc. of absolute alcohol added. The crystals, which separated in radiating masses of thin chrome yellow plates, were filtered off, after standing overnight, and washed with absolute alcohol. On heating, water of crystallization was given off below 100° and the salt became orange in color; at about 190° the crystals sintered and decomposition took place at 212–214°. The preparation contained 10.6 per cent of water of crystallization (theory for mono-salt with $3H_2O$ is 10.32 per cent) and the anhydrous substance contained 14.81 per cent of nitrogen (theory 14.92 per cent). This was therefore approximately pure mono-salt. On account of the difficulty of preparing it this salt has little practical value.

The di-salt, on the other hand, is an extremely useful compound. Provided crystallization is always carried out from a mother liquor containing excess of the reagent and adequate time is allowed, it separates in crystals of constant composition and with a high yield. 1 gm. of histidine dichloride treated with 3.5 gm. of dinitronaphtholsulfonic acid in a total volume of 40 cc. of water gave, on standing overnight at room temperature, material equivalent to 3.252 gm. of anhydrous di-salt, a yield of 94.2 per cent. After evaporation to small volume, the mother liquor slowly deposited 0.12 gm. more, giving a total recovery of 98.2 per cent. Experiments in which mother liquors containing alcohol were employed indicated that no advantage was thereby gained.

Histidine is readily recovered from the di-salt by solution in dilute hydrochloric or sulfuric acid and extraction of the dinitronaphtholsulfonic acid with butyl alcohol, as suggested by

Pratt (4) in the case of the arginine compound. The greater solubility of the histidine compound makes it possible to carry out all except the first extraction at ordinary temperatures. If hydrochloric acid is employed, histidine dichloride may be obtained in a very pure form by concentration. Sulfuric acid, if used, may be quantitatively removed by means of barium hydroxide and free histidine separated in crystalline form by concentration *in vacuo* to a small volume. A very pure preparation can then be obtained by redissolving the crystals in the least amount of hot water, filtering, if necessary, and adding an equal volume of absolute alcohol.

Dinitronaphtholsulfonic acid has already been employed in the examination of a histidine fraction obtained from yeast extract with considerable success. A solution which yielded unmanageable sirups with picric acid gave successive crops of clean material when excess of this reagent was used with very little sirup formation. This investigation is proceeding and results will be published in the near future.

SUMMARY.

Histidine may be almost quantitatively isolated in pure form as the di-salt of 2,4-dinitro-1-naphthol-7-sulfonic acid by crystallization from a solution containing excess of the reagent. The presence of small amounts of mineral acid has little, if any, effect upon the composition or yield of the substance. The di-salt separates in microscopic sulfur-yellow needles which appear to contain ½ a molecule of water of crystallization and which decompose at from 251–254°C. (short stem thermometer) depending on the rate of heating. No change in color occurs below 245°.

This salt presents many advantages over other salts of histidine which have been described not only for isolation but for the purification of histidine. Free histidine or its dichloride may be recovered from the di-salt by decomposition with dilute mineral acid and removal of the reagent by extraction with butyl alcohol.

The mono-salt crystallizes from 66 per cent alcohol containing an excess of histidine in chrome yellow plates which contain 3 molecules of water of crystallization. It becomes orange in color on heating at 100°, sinters at 190°, and decomposes at 212–214°C. It is difficult to prepare and has little practical value.

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XUM

RELATION BETWEEN THE VITAMIN C CONTENT OF A COW'S RATION AND THE VITAMIN C CONTENT OF ITS MILK.*

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(Received for publication, September 30, 1926.)

Up to the present time all the experiments which have been reported dealing with the subject, except those of the authors,[1] have indicated that the vitamin C content of milk varies with the content of this vitamin in the feed from which it is produced. Dutcher, Eckles, Dahle, Mead, and Schaefer[2] found that 20 cc. of summer milk were superior in nutritive value and antiscorbutic potency to 60 cc. of winter milk. Hart, Steenbock, and Ellis[3] state in the summary of their article, "Summer pasture milk is much richer in this nutritive factor [antiscorbutic vitamin] than dry feed milk or winter-produced milk, involving the use in the ration of corn silage or sugar mangels." Barnes and Hume[4] found a seasonal variation in the antiscorbutic potency of cow's milk, while Hess, Unger, and Supplee,[5] by the use of dried milk, found that milk from cows receiving a feed low in vitamin C possessed practically no antiscorbutic properties, while milk from cows on pasture was a fair source of this vitamin.

The authors of this paper began an experiment in the summer

* Contribution No. 128, Department of Chemistry, No. 57, Department of Dairy Husbandry.

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XUM

of 1919 which had for its object the study of the influence of the vitamin content of a diet on the vitamin content of the milk produced by dairy cows. For this work the cows were given a ration consisting of dried beet pulp, pearl hominy (white corn with bran and germ removed), tankage, and bone ash, a ration adequate so far as energy, protein, and minerals are concerned, but very low in the three vitamins, A, B, and C. The experimental cows were allowed to run in a dry lot each day for exercise. As we now know, the sunshine which they received took care of their vitamin D requirement and enabled them to put this vitamin into their milk.

During the 3 years the cows were kept on this experimental feed, the vitamin A, B, and C content of their milk was tested repeatedly by the usual feeding trials. In order to compare the vitamin potency of this experimental milk with that of milk from cows receiving a feed containing these vitamins, parallel feeding tests were run with milk from cows receiving various vitamin supplements, such as carrots, pasture, and fresh green alfalfa. These tests showed clearly that the vitamin A and B content of the milk depended on the vitamin content of the feed, but, in so far as these investigations showed, there seems to be no relation between the vitamin C content of the milk and the feed from which the milk is produced.

In testing for the vitamin C content of this experimental milk, a basal ration consisting of 90 parts of ground oats and 10 parts of dried alfalfa was used for the guinea pigs. In all the tests the guinea pigs were kept in individual cages and each guinea pig was given 30 cc. of milk per day. Records of the weight of the individuals at weekly intervals were kept and a description of the apparent condition of each guinea pig for each day of the test was also recorded. The average number of days before signs of scurvy were observed and the average number of days before the death of the guinea pigs due to scurvy are given in Table I.

This year (1926) a test has been made of the relative vitamin C and D content of milk from cows on pasture, and thus receiving direct sunshine as compared to cows receiving neither direct sunshine nor green feed. Only the results on the vitamin C phase of this experiment are included in this paper.

As the vitamin D content of the two kinds of milk under test was expected to vary due to the influence of the amount of sun-

TABLE I.

	Cow No.	Feed of cows.	No. of guinea pigs.	Average No. of days before signs of scurvy.	Average No. of days before death.
Trial 1, began Feb. 18, 1920.	50 and 60	Experimental feed low in vitamin C.	9	30	52
	Maud.	Regular feed + 10 lbs. carrots per day.	4	24	
	Tipsy.	Regular feed + pasture 35 days after experiment began.	4	23	62
Trial 2, began May 10, 1920.	52	Experimental feed low in vitamin C.	6	39	74
	60	" "	5	39	89
	Maud.	Regular feed + pasture + 35 lbs. fresh alfalfa.	6	23	36
Trial 3, began June 19, 1920.	Maud.	Regular feed + pasture + 40 lbs. fresh alfalfa.	5	20	62
	24	" "	4	26	57
	Tipsy.	Regular feed + pasture.	5	33	63
Trial 4, began Sept. 28, 1920.	60	Experimental feed low in vitamin C.	3	19	38
	52	" "	3	20	34
	Tipsy.	Regular feed + pasture.	3	20	45
Trial 5, began Feb. 3, 1920.	60	Experimental feed low in vitamin C.	6	28	72
	College herd.	Regular winter feed including silage.	4	24	118
Trial 6, began Apr. 16, 1921.	55	Experimental feed for 30 days, then pasture + 10 lbs. fresh alfalfa.	6	25	95
	60	Experimental feed low in vitamin C.	3	23	41
	80	Regular feed + pasture.	3	23	73
	College herd.	" " + "	6	27	45
Trial 7, began July 28, 1921.	55	Experimental feed low in vitamin C.	4	20	47

TABLE I—*Concluded.*

	Cow No.	Feed of cows.	No. of guinea pigs.	Average No. of days before signs of scurvy.	Average No. of days before death.
Trial 8, began May 6, 1922.	60	Experimental feed low in vitamin C.	5	27	65
	55	" "	6	49	96
Trial 9, began Oct. 4, 1922.	55	Experimental feed low in vitamin C.	6	23	37
Trial 10, began Feb. 9, 1923.	55	Experimental feed + 6 lbs. oats sprouted.	13	21	75

shine which the cows received, 1 per cent of cod liver oil was included in the basal ration of the guinea pigs so as to eliminate any possible influence of this factor in the test.

Four cows which would freshen about the same time were placed on this experiment December 1, 1925. Two of these were allowed to run in a dry lot at night but were kept in the barn during the day so they would receive no direct sunlight. The other two were allowed to run with the herd. All four received the regular feed which includes a grain mixture, alfalfa hay, and silage. On March 4, the two which were to receive green feed were placed on pasture. On May 16, the test of the vitamin content of the two kinds of milk was begun. The milk from the two cows kept inside was combined each day and tested as one sample, as was the milk from the two on pasture.

One lot of ten guinea pigs received 30 cc. and another 50 cc. of "inside milk" per day. Two similar groups were used to determine the vitamin C content of the "pasture milk." A lot of ten guinea pigs which received 30 cc. each of boiled milk was run as a check. The effect of the milk on the weight of the guinea pigs and time of the development of the scurvy is shown in Figs. 1, 2, and 3. It will be seen from these figures that there was no significant difference in the antiscorbutic potency of the two kinds of milk.

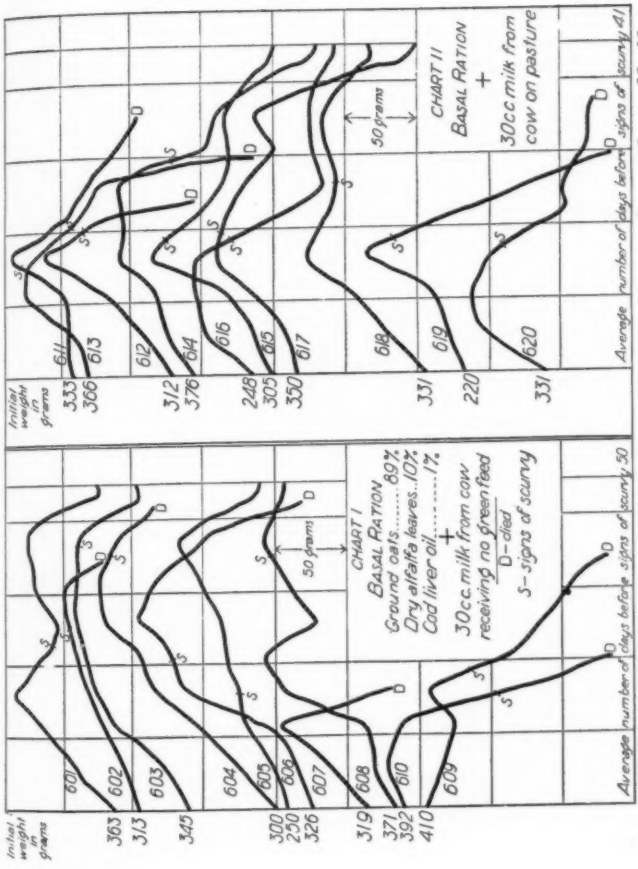
Initial weight in grams
601
602
603
604
605
606
607
608
609
610
363
313
345
300
250
326
319
371
392
410
CHART I
BASAL RATION
Ground oats.....89%
Dry alfalfa leaves..10%
Cod liver oil.....1%
+
30cc. milk from cow receiving no green feed
D-died
S-signs of scurvy
50 grams
Average number of days before signs of scurvy 50
Days
20
40
60
80
90
Initial weight in grams
611
613
612
614
616
615
617
618
619
620
333
366
312
376
248
305
350
331
220
331
CHART II
BASAL RATION
+
30cc. milk from cow on pasture
50 grams
Average number of days before signs of scurvy 41
Days
20
40
60
80
90

FIG. 1.

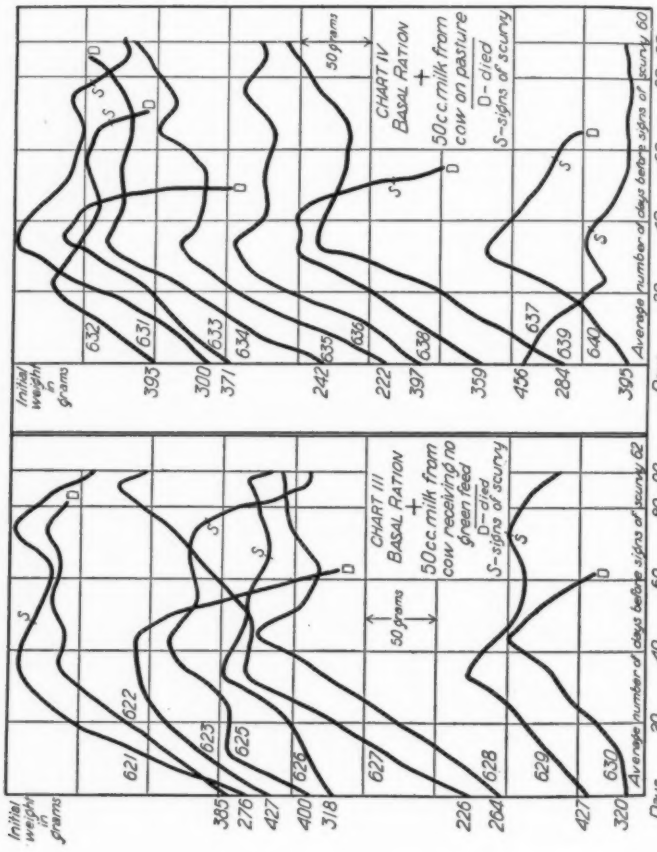
Initial weight in grams
385
276
427
400
318
621
622
623
625
626
627
628
629
630
CHART III
BASAL RATION
+
50cc. milk from cow receiving no green feed
D- died
S- signs of scurvy
50 grams
Average number of days before signs of scurvy 62
Days 20 40 60 80 90
226
264
427
320
Initial weight in grams
393
300
371
242
222
397
359
456
284
395
632
631
633
634
635
636
637
638
639
640
CHART IV
BASAL RATION
+
50cc. milk from cow on pasture
D- died
S- signs of scurvy
50 grams
Average number of days before signs of scurvy 60
Days 20 40 60 80 90

FIG. 2.

These results are in accord with all previous results which the authors have obtained.

Just why these results should differ from those of other investigators is difficult to understand. One possible explanation might be that in the work of the authors already mentioned, there may

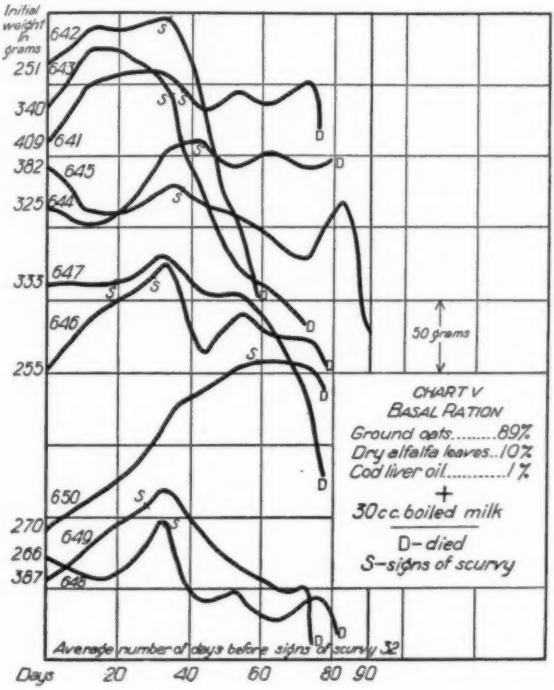

FIG. 3.

have been a variation in the vitamin D content of winter milk and the pasture milk, due to the difference in the amount of sunshine received by the cows. This may have had some influence on the time the symptoms recognized as scurvy began to appear in the guinea pigs. Their results were obtained before the relation of direct sunshine to vitamin D was understood. In previous

work of the authors, the cows receiving the feed low in vitamin C were exposed to as much sunshine as the ones on pasture.

The antiscorbutic potency of the milk produced by Cow 60 (see Table I) after it had been on a feed of kiln-dried beet pulp, pearl hominy, tankage, and bone ash for a period of 3 years, indicates that the dairy cow has, to some extent at least, the ability to synthesize this vitamin. The synthesis of vitamin C by the dairy cow is further indicated by results of experiments which show that they will grow[6] and reproduce[7] on a dry feed which is very low in this vitamin. That certain animals can synthesize vitamin C has been shown by Parsons,[8] who found that livers of rats kept on diets free from this factor contain a large amount of it, as shown by the potency of such livers in protecting guinea pigs from scurvy. Chickens can also apparently synthesize this vitamin, since it is unnecessary to include it in their feed. The synthesis of vitamin C by the dairy cow seems to be about the only explanation of the results reported in this paper.

SUMMARY.

The results obtained in a series of experiments indicate that the vitamin C content of a cow's ration has little if any influence on the vitamin C content of its milk.

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PHYSIOLOGICAL EFFECTS OF DIETS UNUSUALLY RICH IN PROTEIN OR INORGANIC SALTS.*

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With the Cooperation of Helen C. Cannon and Deborah Jackson.

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Plate 1.

(Received for publication, October 18, 1926.)

The experimental investigation of the much discussed problem of the effects of foods exceptionally rich in protein upon various physiological functions has become profoundly modified in recent years by the newly accumulated information with respect to dietary essentials. Before the rôle of the vitamines in nutrition was appreciated the debate regarding the so called high protein régime was fostered either by considerations that were primarily hypothetical in character or by conclusions derived from feeding experiments that would be regarded today as unconvincing, owing to the recognized inadequacies of the rations employed. Thus, from a prolonged experimental investigation, mainly on rats, Watson concluded that a high protein diet is harmful because the growth of the young animal is impaired and in the mature animal certain organs and tissues develop abnormally or become damaged, showing *e.g.* hypertrophy of the thyroid and degeneration of the renal tubules (1). The diets used by Watson were not planned with regard to the possibilities of vitamine deficiencies.

In 1921 we (2) referred to the shortcomings of experiments on record and reported the preliminary results of feeding to rats food mixtures that included an appropriate supply of inorganic salts as well as vitamines A and B, while proteins furnished the almost exclusive source of energy. The animals readily tripled

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

their initial body weight (about 60 gm.) on such diets. Soon thereafter we (3) demonstrated that the rats could grow to at least above 200 gm. of body weight at a good rate even on these extremes of diet that furnished only minimal amounts of the energy in the form of preformed fat or carbohydrate. When the ration was less "one-sided" in composition, yet protein still constituted at least half of its weight, excellent growth was attained; so that we raised the question whether a comparatively high protein content in the dietary during growth is not actually advantageous rather than detrimental as has been suggested by certain writers.

In 1922 Drummond, Crowden, and Hill (4) confirmed our observations in so far as the early good increments of body weight in rats on diets very rich in proteins were concerned. Their young rats nearly trebled their weight before any abnormality was detected; but after about 30 days a retardation of growth ensued. The animals were reported to have maintained excellent health throughout the periods of high protein feeding, although reproduction was not secured on this special diet. The failure of reproduction is, however, easily explained by the absence of fertility-promoting factors in the lack of which both Mason (5) (who has worked with our animals) and Evans (6, 7) and his coworkers have discovered degenerative changes in the testes of rats. Drummond and his coworkers expressed their further conclusions as follows:

> "3. The organs and tissues presented a normal appearance at the end of the experiment and there is little doubt that the abnormal conditions found in similar experiments by other workers were due to causes other than the high protein intake, *e.g.* vitamin deficiency, etc.
>
> "4. The excretion of very large amounts of nitrogenous waste products over considerable periods of time does not appear to cause damage to the kidney in these species.
>
> "5. The failure to grow normally and to reproduce is in our opinion due not to the high protein intake itself but to a lack of balance between this constituent and some other component or components of a normal diet. It is tentatively suggested that the vitamin B, which is believed to be quantitatively concerned in the metabolism of carbohydrates, may likewise be concerned in the metabolism of those non-nitrogenous fragments of the amino-acids which follow a similar fate."

Meanwhile, having observed a striking enlargement of the kidneys in rats that were kept for some time on diets rich in protein (8) we considered some of the histologic aspects presented by the

changes noted. The macroscopic contrasts between the kidneys of comparable animals growing on regimens that differed essentially with respect only to their protein content are illustrated in Fig. 1.

Equally striking are the contrasting sizes of longitudinal sections of the kidneys of these animals (*cf.* Fig. 2).

A review of the data that we have collected on the rate of growth of male rats on diets unusually rich in protein shows that with the highest extremes, although the initial increments of size were as a rule as good as that of our colony on stock rations, there was a tendency to less than normal rates after 200 to 250 gm. of body weight had been reached. Nevertheless the possibilities of reaching a considerable size even on such an extremely unusual diet has been demonstrated by some of the results reported earlier (9). Thus Rat 7735 (Chart 1) attained a maximum of 336 gm. on a diet in which at least four-fifths of the energy was supplied in the form of protein. Such a mixture is evidently not an *ideal* ration. Better results are secured with somewhat lower concentrations of protein, as illustrated by the growth graphs in Chart 5 of the same paper.

With concentrations of protein lower than the above, but still "high" from the standpoint of current popular assumption, our experience has shown an acceleration of growth in the earlier periods that argues for actual advantage over the usual protein content of the ration of growing animals which ranges from 10 to 20 per cent of the calories in the growth food, milk. Of course the statement of food composition in terms of weight percentages of the various foodstuffs may be highly misleading. For purposes of adequate comparison the energy yields, *i.e.* the protein concentrations in percentage of total calories used, should be contrasted. Obviously a high concentration of protein in a fat-rich mixture may be attended with smaller food intake and consequently smaller actual protein intake than in the case of mixtures in which carbohydrates supply more of the non-protein calories.

In the course of observations extended over many years we have repeatedly met with animals that ceased to gain at the usual rate when in the case of males they reached 250 to 275 gm. of body weight. This has happened most frequently on experimental diets representing mixtures of more or less purified food materials rather than on our "stock" rations. The diets referred to were

not unduly rich in protein—the latter usually approximating 18 per cent by weight of the food mixture and 15 per cent of the calories; hence there is no occasion to charge the results in such instances to an undue concentration of protein. We suspect such retardations to be attributable as a rule to dietary deficiencies for vigorous gain in weight has often been resumed when the animals were given a "mixed diet." Our experiences with the increasing (absolute) need of vitamine B as rats grow large, and the cessation of gains when the supply is limited (10, 11), have taught us the significance of quantitative adjustment of small items in the diet. Similarly, growth may become unduly slow when for any reason (such as unexpected deterioration of food) the quantity of vitamine A supplied is unintentionally diminished. Antirachitic factors must also be supplied in some way.

It is on the basis of such suggestions rather than of high concentrations of protein in the ration that we are inclined to explain the uniform failure of Reader and Drummond (12) to secure growth of rats to adult size on "diets containing a high proportion of protein (45–90 p.c.)." 45 per cent of protein in the diets reported by them represents less than half of the caloric intake. Owing to the fact that in our scheme of feeding the vitamine-bearing foods have usually been fed apart from the ration it is not easy to evaluate precisely the calorie distribution between the classes of foodstuffs in the actual intake. For the rations containing 50 per cent by weight of protein with 40 per cent of fat we estimate the protein calories at one-third of the total energy intake. For our foods containing 60 per cent and 70 per cent of protein, respectively, along with some fat and carbohydrate it may range from nearly one-half to two-thirds of the intake. Even with the latter we have secured better growth than Reader and Drummond reported for their 45 per cent protein calories ration. At any rate the successful growths which we obtained have been sufficient to suggest that there is nothing inherent primarily in an intake of at least 30 to 60 per cent of energy in the form of protein that should interfere with the proper increment of weight at a fairly normal, or average rate.

Inasmuch as the difficulties attached to growth on diets unusually rich in protein manifest themselves most outspokenly when the rats attain about two-thirds of their usual adult size we have also tested the capacity of animals raised on lower protein intakes

to adjust to the higher protein levels beginning at stages of growth represented by 250 gm. of body weight or thereabout. Frequently there has been some incipient delay, during which the rats fail to eat satisfactorily and may even lose in weight. Nevertheless adjustment by large animals to protein-rich diets under these conditions is often very satisfactory from the standpoint of subsequent gains of weight and general appearance at least. In other words adjustment to such extremes of diet (representing a food mixture in which proteins supply two-thirds or more of the calorie intake) can still be made at the seemingly "critical" period.

Composition of Blood on Diets Rich in Protein.

It might be expected that diets of the type represented by the food mixtures unusually rich in protein would have some noticeable effect upon the composition of the blood. When, as under the circumstances referred to, the circulating catabolites must contain a proportion of nitrogenous compounds larger than usual, it is conceivable that the latter may exert some physiological or even pathological effect.

The blood of groups of typical rats on diets differing essentially with respect to the proportion of protein calories consumed by them was pooled for analysis with the following results[1]

	per cent	*per cent*		*per cent*	
Protein	80	75		18	Mixed
Fat		10		24	food.
Carbohydrate	16	10		54	
Inorganic salts	4	5		4	
Alfalfa, daily	0.4 gm.	0.4 gm.			
Yeast, daily	0.2 "	0.2 "		0.4 gm.	
Cod liver oil, daily	140 mg.	140 mg.		140 mg.	
Estimated protein calories	79	66		15	(?)
No. of rats	4	5	5	5	4
Body weights, *gm.*	260–350	200–240	260–335	200–219	230–270
Composition of blood per 100 cc.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
Non-protein N	57	44	55	27	31
Urea N	38	28	34	9.2	11

[1] These determinations were made in Dr. Peters' laboratory at the New Haven Hospital.

Jackson (13, 14) has recently reported that "The non-protein nitrogen of the blood of the standard diet rats at the time of death varied from 30 to 40 mg. per 100 cc. whole blood. The non-protein nitrogen of the rats on high protein and high albumen diets varied from 40 to 62 mg. per 100 cc." In no case did an anemia develop.

Urine on Diets Rich in Protein.

In view of the widespread belief that a superabundance of protein in the diet is likely to lead to kidney disorders we examined the urines of a number of rats that had been kept for some time on the following food mixtures consisting of higher or lower concentrations, respectively, of the same protein.

	High protein diet.	"Standard" diet.
	per cent	*per cent*
Casein	75	18
Lard	10	15
Starch	10	54
Inorganic salts	5	4
Butter fat		9
Alfalfa, daily	0.4 gm.	
Yeast "	0.2 "	0.2 gm.

In each case the wire mesh-bottomed cages of the individual animals were placed over large funnels so that the urine could be collected under toluene. The 2 day specimens were invariably faintly acid in reaction to litmus and revealed no abnormal structural sediments. The fluids were filtered and tested for protein with both the heat and Heller's reaction. Faint, though distinct traces were detected in every case; but the reactions were no more noticeable in the case of the animals on the high protein mixtures than those on the "standard" diets. This, too, is in harmony with Jackson and Riggs' (14) experience. Autopsy records showed that on the high protein diets the characteristic renal hypertrophies already described were present; in other words, the diets had actually produced physiological responses in relation to kidney structure as the following typical records indicate:

	High-protein diet.			"Standard" diet.	
	Rat 9831.	Rat 9950.	Rat 9953.	Rat 9833.	Rat 9926.
	gm.	gm.	gm.	gm.	gm.
Observed kidney weight	2.49	1.89	2.13	1.22	1.27
Anticipated kidney weight (according to Donaldson's tables (15))	1.5	1.3	1.12	1.5	1.7

The occurrence of slight albuminuria as a characteristic of the laboratory rat has been reported by others (13, 16). There is no indication from our data of any special nephrotoxic action giving evidence of itself as albuminuria through the high protein diets, even after long periods on such a regimen. The output of urea is greatly increased, as might be expected, so that in carrying out Heller's test on the undiluted urine, crystals of urea nitrate frequently separate in large amounts.

Changes in the Kidneys on Diets Rich in Protein.

We have already called attention to our observation that in rats on diets rich in protein the kidneys usually become strikingly enlarged, the increments in size often exceeding 50 per cent of the normal weight of these organs (8, 9, 17). Marked diuresis occurs on such diets, and the rats drink large amounts of water.

As a standard of comparison for the changes in kidney size we have used the data published in Donaldson's "The Rat," referring them to the total body weight. The autopsies have been performed in almost every case by one of us (P.) Both kidneys are included in the item "kidney weight."

In order to ascertain whether the method of dissection and other procedures employed by us would yield comparable results we have examined a considerable number of animals of different ages and body weights so that our own comparative statistics are available and have convinced us of the propriety of evaluating our findings by reference to the Donaldson data. Table I gives a tabular summary of the kidney weights and other information regarding a group of rats of widely varying size. These animals were maintained on our stock diet, which consists of a specially prepared "dog biscuit" together with an allowance of green vege-

tables, carrots, etc., from time to time. This may be regarded as representative of a so called mixed diet, neither unduly poor nor rich in protein.

TABLE I.

Rat No.	Age.	Body weight.	Kidney weight.	
			Donaldson standard.	Stock food.
	days	*gm.*	*gm.*	*gm.*
B688♂	40	56	0.6	0.68
B568♂	34	75	0.75	0.86
B1232♂	49	96	0.92	0.93
8450♂	59	113	1.05	0.95
8451♂	59	115	1.06	1.03
B1190♂	66	119	1.1	0.96
B1189♂	66	121	1.11	1.06
B52♀	64	124	1.14	1.29
B76♂	75	127	1.16	1.17
B65♀	82	140	1.25	1.1
B647♀	60	157	1.39	1.22
B792♂	67	158	1.39	1.22
9845♀	162	160	1.41	1.33
B800♂	70	160	1.41	1.37
9809♀	155	160	1.41	1.21
B798♂	70	164	1.43	1.28
B1238♂	102	166	1.46	1.12
8299♂	91	173	1.5	0.98
B746♂	87	194	1.66	1.37
8238♂	105	196	1.67	1.11
B570♂	91	205	1.74	1.47
8524♂	128	218	1.84	2.51
B554♂	112	220	1.85	1.42
B1490♀	273	233	1.95	1.38
B1441♀	277	247	2.05	1.75
B1421♀	279	250	2.08	1.85
B1448♀	274	250	2.08	1.53
8239♂	245	290	2.38	1.95
8330♂	225	305	2.5	2.2
B104♂	369	460	3.54	4.46

The data in Tables II and III are derived from animals that have grown for a specified time on so called standard diets consisting of:

	For animals in:	
	Table II.	Table III.
	per cent	*per cent*
Casein	18	
Edestin		16
Salt mixture	4	4
Starch	54	53
Butter fat	9	9
Lard	15	15
Cod liver oil		3
Yeast, daily	0.2 gm.	
" fraction, daily		40 mg.

Comparison of our measurements with the Donaldson data indicates that in the latter the kidney weights referred to body weight tend to be somewhat greater than our own, both in the

TABLE II.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 18 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1287♂	8	41	55	0.58	0.56
B1316♂	8	39	55	0.58	0.56
B1294♂	8	41	58	0.62	0.56
B51♀	39	64	115	1.06	1.05
8343♀	56	86	125	1.14	1.34
8516♂	64	86	148	1.33	1.83
8515♂	64	85	149	1.33	1.46
9805♀	50	158	149	1.33	1.24
B29♀	60	87	162	1.43	1.13
8350♂	59	92	175	1.52	1.37
9833♀	123	228	183	1.57	1.22
8517♂	100	122	193	1.66	1.93
9808♀	115	228	198	1.69	1.14
9810♀	115	228	200	1.7	1.22
9783♀	115	235	202	1.72	1.23
9926♀	106	217	212	1.8	1.27

case of our stock food and the standard foods. Consequently we believe that reference to the Donaldson data will, if anything, put our own measurements to a disadvantage and that the incre-

TABLE III.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Edestin, 16 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8712 ♀	53	73	118	1.09	1.82
8716 ♀	53	77	125	1.14	1.2
8713 ♀	53	73	126	1.14	1.55

TABLE IV.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 95 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8266 ♂	25	95	125	1.15	1.71
8279 ♂	25	100	147	1.31	1.81
8234 ♂	19	106	154	1.37	1.23
8439 ♂	155	185	186	1.61	2.9

TABLE V.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 80 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8442 ♀	72	102	61	0.64	1.45
B1380 ♂	44	203	168	1.48	1.92
8490 ♀	160	191	172	1.5	2.75
8438 ♂	99	139	200	1.7	2.67
B1500 ♂	69	192	210	1.79	2.46
B1536 ♂	44	177	212	1.79	2.2
B1326 ♂	62	244	242	2.02	2.2
8046 ♂	178	329	255	2.11	2.68
8065 ♂	178	322	257	2.13	3.17
B1455 ♂	131	268	260	2.15	2.67
B1523 ♂	146	267	268	2.21	3.76
B1600 ♂	131	253	276	2.28	3.47
B1630 ♂	131	246	286	2.35	3.02
8069 ♂	178	307	287	2.35	3.55
B1501 ♂	142	272	288	2.35	3.03
8052 ♂	178	329	303	2.46	2.95
B1467 ♂	146	268	307	2.5	3.61
B1491 ♂	157	269	322	2.61	3.8
B1610 ♂	146	254	323	2.61	3.95

ments observed by us are represented in minimal terms by such a comparison. Furthermore our own normal statistics, though far less abundant than those rendered available by Donaldson, are recorded in Tables I, II, and III for added comparison. Tables IV to XVII present the statistics for rats that have been fed for varying periods, as indicated, upon diets essentially of the composition given in Table XVIII.

TABLE VI.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 80 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1165♂	51	80	147	1.31	2.04
B1084♂	217	248	185	1.59	2.53
B1158♂	59	84	188	1.62	2.24
B1177♂	55	74	188	1.62	2.19
B1160♂	129	158	257	2.18	2.89
B1141♂	338	367	283	2.35	3.55
B1164♀	336	365	284	2.35	3.36
B1157♂	342	367	325	2.65	3.47

TABLE VII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 80 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8566♂	48	73	134	1.21	1.81
8508♀	161	192	190	1.64	2.14
8418♀	162	189	198	1.69	2.4

It will be noted that in all of the series in which the protein content exceeds 50 per cent of the food by weight there is an almost invariable increment in the size of the kidneys—a gain frequently amounting to 50 per cent, or more, above the "expected normal" kidney weight for animals of that size. In rats fed 50 per cent of casein the increments, if any, appear less striking, while with the smaller quantity (Tables XIII to XV) hypertrophy is not always so noticeable. In the food mixture containing 35 per cent of casein the protein content of the ration represents approxi-

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TABLE VIII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 75 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1594♂	5	42	38	0.46	0.62
B1283♂	8	40	43	0.49	0.86
B1290♂	8	41	44	0.51	0.84
B1282♂	8	40	45	0.51	0.87
B1586♂	3	40	52	0.57	0.66
B1580♂	3	40	55	0.60	0.73
B1668♂	5	42	57	0.61	0.71
B1596♂	3	40	57	0.61	0.85
B773♂	14	35	63	0.65	1.1
B673♂	14	43	64	0.66	1.02
B1624♂	5	44	66	0.69	0.83
B1625♂	3	42	70	0.72	0.69
B40♂	40	65	94	0.9	1.58
B336♂	48	77	94	0.9	1.37
B641♂	28	56	96	0.92	1.42
B347♂	48	77	100	0.95	1.62
B57♂	46	70	105	0.98	2.24
B328♂	48	80	111	1.04	1.63
B662♂	28	56	113	1.05	1.54
B320♂	60	87	129	1.18	1.77
9953♀	111	200	133	1.2	2.13
B599♂	35	56	135	1.22	2.58
B322♂	60	87	141	1.27	1.78
B630♂	42	65	145	1.3	1.68
9816♀	49	162	148	1.33	1.76
9832♀	74	186	148	1.33	1.61
9804♀	44	158	151	1.37	2.25
B628♂	42	65	153	1.37	1.65
B1262♂	42	84	155	1.41	1.65
9950♀	111	200	159	1.4	1.89
B2197♂	60	84	163	1.41	2.16
9831♂	123	228	173	1.5	2.49
B2131♂	61	130	177	1.54	2.00
B725♂	56	80	182	1.57	2.07
9952♀	111	200	195	1.67	2.04
B1442♀	246	272	199	1.7	2.95
B1444♀	246	270	199	1.7	2.92
B1427♂	253	277	209	1.78	2.43
B1489♀	246	269	212	1.78	2.35
B715♂	56	80	212	1.8	2.85

TABLE VIII—*Concluded.*

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 75 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1420♀	246	274	220	1.86	2.66
B1493♀	245	268	222	1.86	2.27
B1363♂	21	163	228	1.93	2.27
B831♂	70	99	230	1.93	2.42
B1249♂	196	238	230	1.93	2.39
B1261♂	205	247	230	1.93	2.22
B1250♂	205	248	239	1.99	2.12
B1251♂	196	239	241	2.02	2.44
9854♂	115	220	249	2.08	3.18
B1180♂	49	197	252	2.12	2.43
B829♂	70	99	260	2.15	2.47
B844♂	37	272	260	2.15	1.87
B1253♂	271	315	263	2.15	2.85
B1419♂	253	277	262	2.15	2.76
B1425♂	256	280	265	2.21	2.47
B1426♂	256	280	271	2.25	2.8
B1436♂	249	276	310	2.54	2.81
B1231♂	293	342	315	2.57	2.88

TABLE IX.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 75 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1055♂	71	99	145	1.3	1.97
B1056♂	71	99	148	1.33	1.85
B803♂	37	69	149	1.33	2.58
B832♂	37	66	166	1.46	2.42
B818♂	37	66	170	1.48	1.95
B833♂	37	66	200	1.7	2.44

mately one-quarter of the total calories. The hypertrophic conditions of the kidney began to be apparent when the protein intake approximated 40 per cent, or more, of the calorific intake. These striking manifestations were not due to any specific effect of casein, which happens to have been the protein used in most of our trials. The kidney changes have been equally striking in

TABLE X.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 60 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1615♂	10	39	33	0.40	0.70
B1562♂	10	38	33	0.40	0.72
B1563♂	10	38	42	0.49	0.84
B1360♂	18	44	84	0.82	1.07
B1367♂	18	44	86	0.84	1.21
B1335♂	45	75	123	1.13	1.32
B1340♂	45	75	129	1.18	1.6
B1383♂	199	233	240	2.02	1.92
B1342♂	199	229	254	2.12	2.23
B1418♂	253	277	312	2.54	2.87
B1324♂	283	313	327	2.65	2.9

TABLE XI.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 55 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8249♂	186	260	290	2.38	2.81
8264♂	160	217	365	2.94	3.47
8250♂	160	241	420	3.33	3.5

TABLE XII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 50 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1124♂	62	86	213	1.8	1.77
B1091♂	63	97	222	1.87	1.93
B1086♂	63	91	235	1.96	2.16
B1087♂	64	94	250	2.08	2.12
B1096♂	66	95	300	2.46	3.23
B1120♂	66	90	311	2.53	2.69
B1101♂	337	365	377	3.04	3.62
B1123♂	333	357	417	3.33	3.42

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TABLE XIII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 35 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1135♂	66	90	240	2.0	2.03
B1132♂	63	87	271	2.24	2.32
B1043♂	70	96	277	2.28	2.17
B2231♂	109	136	285	2.35	2.46
B1046♂	70	96	298	2.44	2.45
B1047♂	119	145	330	2.69	2.67
B1042♂	240	266	337	2.73	2.70
B1133♂	66	90	340	2.75	2.03

TABLE XIV.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 35 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B2162♂	168	191	287	2.35	3.23
B2107♂	192	223	290	2.38	2.83
B2136♂	162	188	294	2.39	3.16
B2106♂	185	216	297	2.42	2.81
B2137♂	162	188	324	2.65	3.4
B2164♂	168	191	373	2.99	3.78
B2161♂	168	191	383	3.04	3.62

TABLE XV.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 35 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B2153♂	162	190	354	2.86	3.41
B2157♂	168	196	375	2.99	3.46
B2154♂	168	196	383	3.04	3.1
B2132♂	162	188	413	3.28	3.31

experiments with "meat residue" fed to the extent of 80 per cent of the diet (Table XVI). Particularly interesting are the hypertrophies observed after feeding large quantities of gliadin amount-

ing to 80 per cent of the food mixture (Table XVII) where the kidney hypertrophy occurred despite the poor gains in body weight owing to the comparative deficiency of the protein in the essential amino acid, lysine.

The period required, on the high protein diets, to bring about the characteristic increments in kidney size is surprisingly brief. Thus in Table VIII there are data giving unmistakable evidence of such hypertrophies within 8 days. After a certain gain in size

TABLE XVI.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Meat residue, 80 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
8430 ♀	68	107	147	1.31	1.92
8513 ♀	196	228	213	1.8	3.5
8440 ♂	104	136	231	1.93	2.75
8441 ♂	210	244	297	2.42	3.67

TABLE XVII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Glisdin, 80 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B530 ♀	26	51	42	0.48	0.95
B422 ♂	54	88	60	0.64	0.96
B441 ♂	42	73	60	0.64	1.1
B463 ♂	42	69	67	0.69	1.02
B402 ♂	54	87	71	0.72	1.07
B405 ♂	42	75	89	0.86	1.28

has been attained the increment does not augment with continuance of the protein feeding; so that as in the case of the body as a whole there are perhaps inherent morphologic factors that limit the possibilities of maximum size.

The compensatory hypertrophy of the remaining kidney after unilateral nephrectomy or unilateral ureteral ligation has been well established (18, 19). In recent experiments by Smith and Moise (20) on rats maintained on our standard diets (containing 18 per cent of protein) the hypertrophy of the remaining kidney

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TABLE XVIII.

Table	For animals in:																
	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII			
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent			
Casein	95	80	80	80	75	75	60	55	50	35	35	35	80				
Meat residue														80			
Gliadin														5			
Salt mixture	5	5	4	5	5	5	5	5	4	4	4	4	5	15			
Starch		15	16		10	10	25		22	37	37	37	15				
Lard				15	10	10	10	37	15	15	15	15					
Butter fat									9	9	9	9					
Cod liver oil								3									
" " " daily, *mg*			140			280			140	140	140			280			
Yeast, daily, *gm*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4	0.2	0.2	0.4	0.2	0.2	0.2			
Alfalfa, " "	0.4	0.4	0.4	0.4	0.4		0.4		0.4	0.4		0.4	0.4				

after a unilateral nephrectomy was never observed to exceed about 80 per cent of the estimated original weight of a single kidney. Studies of the effect of high protein feeding upon rats with a single remaining kidney are in progress.

Referring to hypertrophic rat kidneys after complete ligature of the opposite ureter for periods of 1 to 600 days duration Hinman (18) states:

> "The hypertrophic changes as measured by an increase in the size of the kidney were complete on an average of twenty to thirty days. There was no demonstrable increase in size beyond this period, even of those kidneys doing double duty for almost two years. In no case was the size of the kidney doubled nor did the increase ever even approach to this degree."

This investigator states that doubling the demand upon the kidney never doubles its size, and he intimates that an overwhelming demand may actually lead to atrophy of the kidney. We have never found this to occur under the conditions of our experiments. Hinman's figures show that the increase in the size of the rat kidney is not completed until between the 28th and 42nd days (21). In the rabbit after nephrectomy Addis, Myers, and Oliver (19) observed a compensatory hypertrophy not exceeding 32 per cent in the weight of the remaining kidney.

In view of the reports that have been current regarding the genesis of nephritis and sclerotic changes (22–25) as a result of diets relatively rich in protein, special attention was devoted to possible evidences of kidney damage in the many rats examined post mortem, after various periods of high protein feeding.

In comparison with our experience the degenerative changes reported by Polvogt, McCollum, and Simmonds (22) in the kidneys of rats maintained on diets considerably lower in protein concentration than many of those in our own series, were surprising. Their experiments differed in two respects; namely, the make-up of the ration, which consisted almost entirely of natural foods, and the prolonged duration of the feeding trials whereby the animals were kept well into their 2nd year. Accordingly we have repeated the experiments, duplicating the diets as nearly as possible, the food mixture consisting of:

	per cent
Wheat	30
Corn	20
Casein	20
Liver	20
Navy beans	7.5
$CaCO_3$	1.5
NaCl	1

Male rats were kept on these diets, estimated to contain about 40 per cent of protein, for 400 days or longer. The rate of growth of these animals was excellent. Of six fed as described, three showed some hypertrophy of the kidneys, while the rest gave no indication of unusual kidney size. Furthermore, in order to control the old age factor we secured, through the kindness of Professor H. C. Sherman of Columbia University, a group of old animals 500 days or more of age which had been fed on his routine stock diet that may be regarded to have the usual moderate concentration of protein. These animals were intended to serve as "controls" with respect to age.

The rats on a diet very high in protein showed some roughness and dryness of the coat and a diminished amount of subcutaneous fat, the lack of which caused skinning of the animal to be difficult. There was, however, considerable fat in the groins, abdomen, and about the testes. The fat was always pale. The thymus gland was atrophied. In many of the rats on the mixtures of purified food substances the testes were underdeveloped, a fact probably explained by a dietary lack of the vitamine necessary for development. The kidneys were invariably found to be hypertrophied. The hypertrophy was obvious the moment that the kidneys were exposed to view, for they appeared not only abnormally long and wide, but abnormally plump in comparison with the kidneys of the control rats. No constant variation was noted in the spleen, liver, adrenal glands, or pancreas. The thyroid gland, parathyroids, and hypophysis were not examined. No changes were noted in the skeleton. On gross examination, therefore, the rats on the diets high in protein showed no differences from rats on ordinary diets, with the exception of hypertrophy of the kidney and poorer states of nutrition.

The kidneys from 200 rats were selected for histological study. 92 of the animals had been on high protein diets—from 35 to 95 per cent in protein—for 7 to 400 days. The oldest animals of this high protein group, numbering six, had been fed the Polvogt, McCollum, and Simmonds (22) diet from weaning for approximately 400 days; one animal had received the protein in the form of meat residue—95 per cent of the diet—and six in the form of gliadin—80 per cent of the diet. The protein in the diet of all the others

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was casein, ranging in quantity from 35 to 95 per cent. The great majority of the animals were weaned to the high protein diets, but fifteen were first placed upon them when 60 to 180 days old. The control rats, numbering 50, were not recruited from the same litters as the rats on the high protein diets, but corresponded roughly in age and weight. They were fed in different ways. One group of sixteen rats was maintained on a diet containing 18 per cent casein for 8 to 262 days; and another group of twenty-six rats on the stock diet of dog biscuit and cabbage for 20 to 450 days. Eight animals had been kept on the Sherman diet for 490 to 540 days. The animals in the group just mentioned were considerably older than the oldest rats on the high protein diets. In addition, the kidneys of a miscellaneous group of 58 rats were studied; the majority had been on diets of average protein concentration but containing large quantities of various test substances, *e.g.* urea, monosodium phosphate, disodium phosphate, sodium chloride, the Osborne-Mendel salt mixture. The diets fed to the other rats of this miscellaneous group were characterized by a very low protein content well under the limits of protein requirement but otherwise well constituted.

The kidneys were fixed in formalin and stained with hematoxylin and eosin.

In general the kidneys of the rats which received diets containing very large proportions of protein, 70 per cent or more of the total ration, differed in certain histological details from the kidneys of the control rats on the lower protein diets; in the former the epithelium of the tubules seemed larger and the tubules themselves dilated; also, the inclusions of tubular epithelium in the glomerular capsules appeared to be larger and more numerous.

Since accurate measurements of the tubular epithelium could not be made, it was necessary, in forming the opinion that the epithelium was higher in the tubules of the experimental rats, to depend upon impressions obtained by comparison of sections under the microscope. This was exceedingly difficult. We can say merely that in the case of many of the rats kept for 50 days or more on diets extremely rich in protein the cells of the convoluted tubules looked unusually large, larger than in the control animals; that the apices of the cells extended far out from the basement membrane; and that the histological structure of the cortex in general seemed the more compact, presumably due to the increased size of the cells themselves. Differences in the size of the tubular epithelium, however, were not sufficiently striking to enable us to distinguish with accuracy the sections of the animals on the high protein diets.

The lumina of the tubules were considerably dilated in the rats kept on the extremely high protein diets for 50 or more days. In general the dilatation was conspicuous in the midzone of the kidneys and was more marked in certain parts than in others. Where it existed, however, it was fairly uniform, in contrast to the dilatation found in the old control rats which seemed to involve tubules here and there. The dilatation was exceedingly well marked in two rats aged 66 days which had been on the diet of 75 per

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cent casein for only 30 days. In the case of these two animals the glomerular sacs were also greatly distended and contained much pink-staining cellular debris; the tuft occupied only one-half or two-thirds of the capsular space. In many rats on the very rich protein diets the dilatation of the tubules was marked enough to enable us to guess correctly at a glance the high protein nature of the feeding. Dilatation of the tubules was not present or at least not clearly recognizable in the sections of the other rats which were either on the high protein diets for short periods, or else on diets in which the proportion of protein was not particularly large.

The extension of tubular epithelium into the glomerular capsule must be regarded as a normal condition in the rat, because in the majority of control animals one glomerulus at least could be found which contained traces of it. With very few exceptions in the rats on the high protein diets the inclusions were numerous and thick, occupying a fifth to a third of the intracapsular space. The thickness of the inclusions was due apparently to two factors: the tubular inclusion was an invagination and hence was composed of double layers of cells; the invaginated epithelium was thick, resembling that of the convoluted tubule. Perhaps one of the best evidences that the tubular epithelium in the experimental animals was larger than in the control animals was afforded by the fact that the glomerular inclusions in the former were composed of thicker epithelium. Normal epithelium of Bowman's capsule could be seen underlying the epithelium of the inclusion. Proliferation of the capsular epithelium was not found in any animal. The presence of numerous large inclusions of tubular epithelium was so characteristic as to be almost pathognomonic of high protein feeding.

At first we were under the impression that the glomeruli were the larger in the rats on the high protein diets. The glomeruli in the very old rats on the Sherman diet, however, were among the largest seen. The question of the relative size of the glomerulus in rats on high protein diets must be left for subsequent investigation.

In addition to the changes thought to be of a more or less physiological nature some rats on the high protein diets showed actual lesions of the kidneys. In certain of the control animals, however, lesions of an exactly similar nature were found; they were focal and involved the tubules alone, or tubules and glomeruli, and in some instances were characterized in addition by infiltrations of small round cells.

The lesions of the glomeruli consisted in thickening of the basement membrane which in most instances was accompanied by fusion of the membrane with the glomerular tuft. There were two kinds of alterations in the tubules: some were greatly distended so as to be several times the normal size and filled with a cast-like material; the epithelium was in many instances quite flat. Other tubules, apparently abnormal, contained no casts and were easily recognizable in the hematoxylin- and eosin stained sections by the intensity of the blue stain. The latter feature was due to the fact that the nuclei which were large and stained pale blue were so numerous as to meet or even overlap and in large part hid the cytoplasm from view.

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TABLE XIX.

Data Concerning Those Rats on High Protein Diets which Showed Focal Lesions in Kidneys.

Rat No.	Diet.	Protein.	Initial age.	Duration of experiment.	Final age.	Degree of kidney hypertrophy.	Focal lesions.
		per cent	*days*	*days*	*days*		
B1101	Casein.	50	29	335	364	++	Scattered small lesions of tubules.
B1123	"	50	24	332	356	0	A single small lesion of tubule.
B1324	"	60	30	286	316	+	Very slight lesion of tubule.
B1420	"	75	28	249	277	+++	Several small lesions of tubules with cast formation.
7689	"	80	26	235	261	++++	Several dilated tubules with large cast formation and some round cell infiltration. Several glomeruli found with thickened capsule and adherent tuft.
B1157	"	80	25	341	366	+++	Scattered dilated tubules with cast formation. One glomerulus with thickened capsule and adherent tuft.
B1164	"	80	29	338	367	+++++	A few scattered minute lesions characterized by dilatation of tubules with cast formation.
B1380	"	80	159	44	203	++	One small lesion of tubule with cast formation.
B1467	"	80	122	146	268	++++	" " " " "
B1610	"	80	108	146	254	+++++	Minute lesions of tubules.
7583	"	95	32	288	320	+++++	Two minute lesions with cast formation.
7797	"	95	33	297	330	++++++	A few minute lesions of tubules with cast formation.
B1401	Polvogt, McCollum, Simmonds.	40	28	402	430	0	Several small " " " " " "
B1408	" "	40	31	402	433	++	Several areas characterized by round cell infiltration, lesions of tubules with cast formation. Several glomeruli with thickened capsule and adherent tuft.

							lesions of tubules with cast formation. Several glomeruli with thickened capsule and adherent tuft.
B1409	Polvogt, McCollum, Simmonds.	40	31	402	433	+	Numerous focal lesions some of which are large, consisting of abnormal tubules with cast formation and fibrosing glomeruli with and without round cell infiltration.
B1443	" "	40	33	400	433	++	A few very minute focal lesions of tubules with and without cast formation. A few glomeruli with thickened capsules.
B1446	" "	40	31	400	431	0	Numerous lesions of tubules with large cast formations. One glomerulus with thickened capsule and adherent tuft.
B1452	" "	40	30	400	430	−	Numerous small lesions of tubules with and without cast formation. One glomerulus with thickened capsule and adherent tuft.

The latter, however, also had a more definitely blue color than the normal epithelium. Some of these tubules had large lumina and thin epithelium but others had greatly contracted lumina with or without thickened basement membrane; or else the lumen had disappeared altogether and the nuclei heaped on each other formed a thick border to the pale center of cytoplasmic substances. It was common to find the abnormal tubules just described and the dilated tubules containing casts in combination. In many focal lesions, particularly in those found in the old rats, abnormal glomeruli or accumulations of round cells were present.

The rats on the high protein diets showing these focal lesions are listed in Table XIX.

It will be seen that the youngest animal in the high protein group showing a focal lesion was 203 days old; the average age was 348 days. In regard to the percentage of protein in the diet, eight of the eighteen rats were on diets of 50 per cent or less of protein. These eight animals, however, were very old. The youngest animals showing focal lesions were on diets containing 80 per cent protein. Fifteen of the animals showing lesions had been on the diet since weaning, three having been put on the diet when over 100 days old. All the animals on the high casein diets having focal lesions showed marked dilatation of tubules, and most of them certainly seemed to have unusually large cells in the tubules. There seemed to be no relationship between the focal lesions and degree of hypertrophy of the kidney.

The remaining eighteen rats on very rich protein diets—(eight on 95 per cent, six on 80 per cent, three on 75 per cent, one on 60 per cent casein for 200 to 360 days)—showed no focal lesions whatsoever in the kidneys.

As will be seen by examination of Table XIX, the focal lesions themselves were exceedingly slight in the entire group, with the exception of five on the Polvogt, McCollum, and Simmonds diet in which they were severe, and in two on the casein diets in which they were moderate. The renal tissue apart from the focal lesions in all these animals seemed to be in a healthy condition.

Turning now to the consideration of the focal lesions in the control rats, the kidneys of only five were affected. These five animals were all on the Sherman diet and were 515 days old or older. The lesions shown exactly corresponded to those already described in the rats on the Polvogt, McCollum, and Simmonds diet, which also were very old. Sixteen rats on the average protein diets for more than 200 days—217 to 509 days old—showed no focal lesions.

Microscopic examination of the kidneys of the rats on otherwise unusual diets, *e.g.* diets to which salts and urea had been added, failed to disclose any abnormalities.

In our study and interpretations of the histological pictures of the kidneys we have been greatly influenced by the unpublished observations of our colleagues, Drs. T. S. Moise and A. H. Smith, who are engaged in investigations of the renal changes after unilateral nephrectomy in rats on high

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protein diets. The publication of their report should have preceded ours. Until we became conversant with their work, we had failed to perceive that the dilatation of the tubules in our animals on the extremely rich protein diets was an effect of the high protein content and not a chance condition, or to observe that the inclusions of tubular epithelium in the glomerular capsule were so much more common in the rats on the high protein diets than in the control rats on the average diets. Furthermore, we had not appreciated that the focal lesions occurred in the rats on the high protein diets at an earlier age than in the rats on the control diets. Previously we had explained the focal lesions in both control and experimental animals as the result of the factors included under the term "age." Now we do not know whether the earlier appearance of these focal lesions in the rats on the high protein diets is to be explained by the concentration of protein in the diet in a specific sense or by the increased load of an unbalanced ration.

Jackson (13, 14) also reports inability to produce nephritis of any sort in rats by high protein diets over a long period of time.

We have attempted, further, to ascertain whether the renal hypertrophies induced by feeding protein in large amounts are followed by retrogression or "compensatory atrophy" when the diet is changed to one of a lower protein concentration. The problem has been complicated by the circumstance that some growth may have been in progress while the possibilities of reduction were presented, so that in some cases the kidneys may have "grown up" to their previously hypertrophied size during the interval of low protein diet; or the impulse of growth may have compensated the tendency to reduction in size. Some results of decreasing the dietary protein after rats had been on a high protein régime that might be expected on the basis of our experience to bring about renal hypertrophy are shown in Table XX. The data under "duration of experiment" show the period of high protein and lower protein feeding respectively, the kidney weights being compared with those "expected" for a rat of the same body weight. The protein concentrations of the diet for the two periods are also indicated. The tendency to retrogression in size, or at any rate the failure to maintain the undoubted earlier hypertrophic gains, is evident.

Analysis of the data secured has not yet disclosed any striking proportionality between the protein intake and the degree of renal hypertrophy in so far as comparisons between "high protein" and "very high protein" are concerned. One gains the impression

TABLE XX.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Casein, 75 per cent, 18 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B1472♂	14 7	59	112	1.05	1.11
B1483♂	14 9	64	136	1.23	1.6
B1525♂	14 7	58	146	1.31	1.24
B1485♂	14 7	59	148	1.33	1.78
B1470♂	14 11	64	160	1.41	1.44
B1468♂	14 11	64	170	1.48	1.36
9819♀	56 62	231	201	1.7	1.28
9951♀	52 62	203	205	1.75	1.79
9803♀	63 62	233	225	1.89	1.47
B1054♂	71 47	146	240	2.02	1.89
9966♀	52 62	193	245	2.04	1.43
B1109♂	71 68	163	241	2.02	2.15

that the increments in kidney size are limited to between 50 and 100 per cent of the "normal" weight of the organs. Perhaps this represents a limit placed by the structural or morphologic factors

of the kidney; indeed, it is conceivable that food intake and consequent possible gain in weight on foods very rich in protein are limited by the possibilities of physiological renal response.

The gains in kidney weight are not due merely to retained urine. The histological observations speak against such an hypothesis; and chemical examinations show that the enlarged kidneys are not unduly rich in water. Thus we have found:

Rat No.	Protein content of diet.	Kidney composition.	
		Dry solids.	Water.
	per cent	*per cent*	*per cent*
B1262	75	24.3	75.7
B1238	About 15	25.8	74.2

The attempt to secure retrogression in kidney size through feeding the lowest concentrations of protein compatible with mere maintenance of body weight failed to bring any noteworthy reduction in the weight of the organs. Various proteins at differ-

TABLE XXI.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Lactalbumin, 5 per cent to 10 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B311♂	106	188	170	1.48	1.66
B111♂	138	218	205	1.74	1.96
					Casein, 6 per cent.
B242♂	89	198	164	1.43	1.14
B267♂	89	197	178	1.54	1.42
B282♂	89	194	188	1.62	1.49
B244♂	89	197	190	1.64	1.34
B262♂	89	191	209	1.77	1.38
					Casein, 8 per cent.
B492♂	173	282	183	1.59	1.28
B465♂	202	318	207	1.76	1.45
B468♂	173	289	247	2.05	1.64
B444♂	202	319	264	2.18	2.29

ent levels of intake were tested. Some of the results are detailed in Table XXI. The food mixtures included starch 61 to 66, lard 21, inorganic salts 4, cod liver oil 4 per cent, together with appropriate daily doses of yeast or yeast concentrate. The character and content of protein used is indicated in the table.

TABLE XXII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Salts, 12 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B35 ♀	60	85	132	1.19	1.00
B37 ♀	116	141	173	1.5	1.16
9703 ♀	63	192	178	1.54	1.32
9600 ♀	120	265	189	1.62	1.47
B33 ♀	60	87	196	1.67	1.42
9640 ♀	58	196	201	1.72	1.38
B41 ♂	117	137	210	1.79	1.3
B46 ♂	117	141	268	2.21	1.8
					Salts, 17 per cent.
B717 ♂	50	78	107	1.0	0.96
B695 ♂	50	80	133	1.2	1.56
B698 ♂	50	80	138	1.24	1.25
B726 ♂	50	78	141	1.27	1.4
B722 ♂	50	78	158	1.39	1.18
					Salts, 17 per cent to 22 per cent.
B838 ♂	46*	80	93	0.9	1.09
B845 ♂	46*	80	97	0.93	0.99
B834 ♂	46*	77	111	1.04	1.07
B839 ♂	46*	80	119	1.1	0.99
B826 ♂	46*	77	125	1.15	1.24

* 17 per cent salts first 7 days of experiment.

If the renal hypertrophy were merely the expression of an adaptation of the kidneys to a larger task of excretion it might be expected that an increment in any dietary component that leads to catabolic products eliminated by these organs would bring about an enlargement of these structures. The problem is, however,

apparently not quite so simple; for largely increased intake and consequent renal elimination of a variety of inorganic salts has not in our experience been productive of hypertrophy even where the salt content of the diet has been augmented to the highest extent compatible with successful continuance of the feeding tests.[2] We have already made tests with concentrations of our usual complete salt mixture (26) up to 22 per cent of the diet (Table XXII); with inclusions of 18 to 25 per cent of sodium chloride in

TABLE XXIII.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	NaCl, 18 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B2089♂	35	66	75	0.75	0.88
B2090♂	35	66	80	0.80	0.87
B2091♂	42	73	85	0.84	1.03
B2115♂	34	62	86	0.84	0.96
B2051♂	35	64	87	0.85	0.85
					NaCl, 20 per cent.
B1890♂	42	66	55	0.60	0.88
B1888♂	42	66	75	0.75	1.15
B1881♂	74	100	78	0.75	0.98
B1882♂	49	75	83	0.82	1.05
					NaCl, 25 per cent.
B1812♂	10	41	37	0.44	0.68
B1859♂	10	37	37	0.44	0.86
B1790♂	10	45	38	0.47	0.58

our standard diet (Table XXIII); with additions of sodium acetate, ammonium chloride (producing tendency to acidosis), ammonium acetate, and acid sodium phosphate (Table XXIV). The com-

[2] Needham, J., (*Biochem. J.*, 1924, xviii, 891) working with rats, has fed diets in which the salt content of the food was as high as 17 per cent over long periods, whereby a vigorous polyuria was produced. He has described degenerative changes in the kidneys; but it is not entirely clear that these are attributable solely to the diet.

position of the diets of the animals in these experiments is given in Table XXV.

In view of the outcome of these tests in which the results of feeding high concentrations of protein was in marked contrast to

TABLE XXIV.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	$NaC_2H_3O_2$, 2.5 per cent.
	days	*days*	*gm.*	*gm.*	*gm.*
B772♂	46	71	128	1.16	1.17
B713♂	57	81	148	1.31	1.3
B763♂	47	72	154	1.37	1.3
					NH_4Cl, 3 per cent.
B967♂	83	143	196	1.67	1.19
B902♂	76	140	215	1.82	1.76
B957♂	76	136	223	1.87	1.5
					$NH_4C_2H_3O_2$, 3 per cent.
B1010♂	76	121	208	1.77	1.65
B1003♂	76	128	214	1.82	1.75
B1012♂	84	128	338	2.73	2.46
					H_2NaPO_4, 3.2 per cent.
8466♂	38	64	97	0.93	0.75
8468♂	38	64	102	0.97	0.86
8467♂	38	64	113	1.05	0.98
8470♂	38	64	119	1.1	1.06
					H_2NaPO_4, 7 per cent.
B1945♂	62	89	103	0.97	0.91
B1913♂	63	91	124	1.13	1.37
B1949♂	63	90	126	1.15	1.9
B1911♂	63	91	127	1.16	1.48

the failure to produce similar hypertrophy through the feeding of liberal amounts of various salts, it seemed highly probable that urea, derived from the protein, might be the specific agent responsible for the kidney enlargements. Early tests with inclu-

TABLE XXV.

	For animals in:										
	Table XXII.			Table XXIII.			Table XXIV.				
	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent
Casein	18	18	18	18	18	18	18	18	18	18	18
Salt mixture	12	12	12	4	4	4	4	4	4	4	4
NaCl		5	10	18	20	25					
$NaC_2H_3O_2$							2.5				
NH_4Cl								3			
$NH_4C_2H_3O_2$									3		
H_3NaPO_4										3.2	7
Starch	50	45	40	40	38	33	48.5	48	48	50.8	47
Lard	11	11	11	11	11	11	24	24	24	15	15
Butter fat	9	9	9	9	9	9				9	9
Cod liver oil							3	3	3		
Yeast, daily, gm	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

TABLE XXVI.

Rat No.	Duration of experiment.	Final		Kidney weight.	
		Age.	Body weight.	Donaldson standard.	Urea, 18 per cent.
	days	days	gm.	gm.	gm.
B1392♂	49	75	85	0.84	0.88
B1272♂	56	95	105	1.03	1.13
B1268♂	56	95	107	1.03	1.17
B1351♂	56	91	118	1.11	1.37
B1354♂	56	91	143	1.27	1.42
B1270♂	84	123	159	1.41	1.44
B1266♂	84	122	160	1.41	1.47
					Urea, 22 per cent.
B709♂	35	59	76	0.76	1.08
B689♂	35	68	93	0.9	1.32
B1669♂	63	94	95	0.92	1.27
B1671♂	53	84	97	0.93	1.15
B687♂	35	68	97	0.93	1.13
B1658♂	39	71	115	1.06	1.18
B1350♂	59	94	144	1.29	1.68
B1359♂	71	106	174	1.52	1.6
B1353♂	71	106	182	1.59	1.62
					Urea, 28 per cent.
B1755♂	38	74	69	0.72	1.01
B1765♂	35	70	78	0.79	0.83
B1795♂	50	85	116	1.08	1.18
B1818♂	50	83	122	1.13	1.14

sions of preformed urea in the diet to an extent comparable with what might be expected to be formed from the high protein diets gave indications of the induction of corresponding renal hypertrophies. For the most part, however, concentrations of urea in the diet up to the limit of tolerance of the rats for the foods containing this nitrogenous catabolite have not produced the expected hypertrophies. Some of the data are included in Table XXVI. The composition of the food mixtures was essentially as follows: casein 18, starch 54, lard 24, inorganic salts 4 per cent, together with 280 mg. of cod liver oil and 0.2 gm. of yeast daily.

SUMMARY.

A renewed study of the possibilities of growth, in the albino rat, on diets exceptionally rich in protein has confirmed our earlier observations on large increments of body weight at essentially normal rate and to adult size under such dietary conditions. The failure of some other investigators to secure equally good growth is ascribed, in the absence of more explicit information, to possible inadequacies of the rations employed in their feeding trials. In some of our successful experiments the protein component of the dietary represented two-thirds, or more, of the entire calorie intake. Animals raised to large size on diets of a relatively low protein concentration can also become adjusted to the protein-rich diets.

The blood of the animals on a protein-rich regimen shows a somewhat higher content of non-protein nitrogen than is found in the case of rats living on the usual laboratory diets. Comparative data are also given for the urea nitrogen of the blood.

The urine of animals on diets extremely rich in protein showed no abnormalities indicative of chronic renal disorders attributable to the protein.

Hypertrophy of the kidneys is a characteristic feature of the animals on a diet in which the protein intake exceeds about one-third of the food fuel of the ration. This renal response is characteristic for a variety of dietary proteins tested. The histological features of the enlarged kidneys are described. The hypertrophy has been produced by ingestion of various proteins. The period required to bring it about is surprisingly brief. Retrogression in kidney size by suitable changes in diet after hypertro-

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phy has been induced by protein feeding has also been investigated. The possibility of comparable renal hypertrophy through ingestion of various substances—urea, inorganic salts—normally excreted by the kidneys has also been studied in some detail. Urea, fed in amounts equivalent to that derived from effective quantities of protein, failed to bring about an equally noticeable renal enlargement.

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EXPLANATION OF PLATE 1.

FIG. 1. Showing effect of high protein diet on size of kidneys.

Rat B833♂.	Rat B746♂.
75 per cent protein.	Stock diet.
Body weight 200 gm.	Body weight 194 gm.
Kidneys weigh 2.44 gm.	Kidneys weigh 1.37 gm.

FIG. 2. Showing comparable longitudinal sections of kidneys of animals in Fig. 1.

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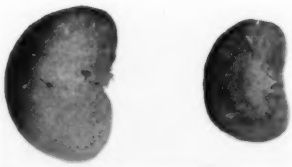
FIG. 1.



Rat B 833♂. Rat B 746♂.

FIG. 2.

(Osborne, Mendel, Park, and Winternitz: Diets rich in protein.)

LIPID EXCRETION.

IV. A STUDY OF THE RELATIONSHIP OF THE BILE TO THE FECAL LIPIDS WITH SPECIAL REFERENCE TO CERTAIN PROBLEMS OF STEROL METABOLISM.*

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Previous investigations (1, 2) have demonstrated that there is an excretion of lipids in the feces independent of the diet. It appeared desirable to study this excretion further with reference to its origin, not only because such a study might add to our rather meager knowledge of the functions of the intestine as an organ of excretion, but also because of the probable bearing of the results on questions of lipid metabolism. In reviewing various possible sources of the fecal lipids one thinks of the bile immediately because of the widely accepted theory that it serves as the pathway of cholesterol excretion (see below) and because of its close connection with fat absorption. Indirect evidence presented in a preceding paper (1) indicated that the bile is not the source of the fecal lipids. It was the purpose of the present investigation to obtain further data which might determine more conclusively what part, if any, the bile plays in lipid excretion. It seemed logical that such definite information would be furnished by a comparison of the fecal lipids before and after exclusion of the bile from the intestine. Should the excretion be found to be the same in amount and composition under the same dietary conditions before and after operation, it would seem reasonable to conclude that the bile had nothing to do with the fecal lipids. The plan of experiment, therefore, was quite simple. Several dogs, which

* A preliminary report of this investigation was published in the Proceedings of the Society for Experimental Biology and Medicine for May, 1926.

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had been used in previous work with lipid-free diets, were available. Various operations designed to exclude bile from the intestine were performed on these animals and their fecal lipids were studied under the same experimental conditions employed while they were still normal.

The work has been of particular interest because of its bearing on certain problems of sterol metabolism. Of these one of the most important has been that of determining the source and purpose of the cholesterol of the bile.

The old view of Naunyn (3) and his school that the cholesterol of the bile is elaborated by the mucosa of the biliary passages has been pretty much abandoned in more recent years by the majority of authors (Bacmeister and Havers (4), Beumer (5), Campbell (6), Cammidge (7), Chauffard, Laroche, and Grigaut (8), Dorée and Gardner (9), Grunke (10), Kusumoto (11), Liang and Wacker (12), Röhmann (13), Stepp (14), Thannhauser (15), Rothschild and Rosenthal (16), and Nathan (17)), in favor of the hypothesis that it is secreted by the liver as a waste product for excretion through the feces. There is indeed considerable evidence to indicate that the bile represents the principal pathway of cholesterol excretion. Bang (18), Beumer and Bürger (19), Schmidt (20), and Fasiani (21) have found cholesterolemia in cases of obstructive jaundice while Chauffard, Laroche, and Grigaut (22) have found an increase in blood cholesterol after ligature of the common duct. McClure and Vance (23) have shown lower cholesterol in the duodenal contents of patients with various pathological conditions of the liver and bile ducts than in normals. Evidence for a parallelism between blood and bile cholesterol has been obtained by Adler (24), Bacmeister and Havers (4), and Rothschild and Rosenthal (16). An increase in the cholesterol of the bile in the case of a single dog after injection of toluenediamine was found by Kusumoto (25), who thought the extra cholesterol came from destruction of red blood cells.

On the other hand it has been shown by Bacmeister and Havers (4) that there is not necessarily a linear relationship between blood and bile cholesterol by the finding that in pregnancy the bile cholesterol decreases as that of the blood increases. Moreover Stepp (26) has had five cases with complete obstruction of the bile duct, of which only two had high blood cholesterol and in one the amount was below normal. Ssokoloff (27) tied off the bile ducts in three dogs and concluded that there was a definite rise in blood cholesterol; but Hueck (28) points out that the increases obtained were within the experimental error of the method. The evidence derived from comparison of food and bile cholesterol is still less convincing. Goodman (29) and Bacmeister (30) have shown a rather questionable relationship between the protein of the diet and the bile cholesterol, while Goodman has further shown that there is no more cholesterol in the bile after feeding brains, containing over 2 per cent cholesterol, than after feeding egg white

containing traces. D'Amato (31) has found a definite increase in bile cholesterol after high cholesterol feeding but Whipple (32) points out that with an estimated cholesterol intake of 5 to 6 gm. per day the increase in bile cholesterol was about 2 mg. D'Amato himself feels that the bile is a very small factor in cholesterol excretion. McMaster (33) has recently reported a considerably larger influence of food on bile cholesterol, finding a decrease of 35 mg. per day on fasting and an increase of 57 mg. per day on feeding 200 gm. of ox brains and three eggs in addition to the regular mixed diet. Though the amount in the diet was not determined, it is evident that the increased cholesterol of the bile represents but a minute fraction of the increase in the food. Fasiani (34) has reported increases in bile cholesterol after intravenous injection but Whipple (32) points out that the rise is but 8 mg. per day after a dose of 1.7 gm. Thomas, Kausch, and Jankau (quoted from Nathan (17)) found independently that cholesterol introduced into the body either *per os* or parenterally was absorbed without any increase in the cholesterol of the bile.

The evidence obtained in the present investigation, in agreement with that mentioned in the preceding paragraph, tends to minimize the importance of the bile as a pathway of cholesterol excretion, and in fact it is rather indicated that under some conditions at least, little, if any, is thus removed from the organism.

A second, very closely related question of cholesterol metabolism has concerned the possible reabsorption of cholesterol reaching the intestine by way of the bile. On the basis of rough calculations Dorée and Gardner (35) reached the conclusion a number of years ago that a considerable portion of the cholesterol of the bile must be reabsorbed by dogs because only about one-fifth of that calculated to reach the intestine by way of the bile was found in the feces. Beck and Wacker (36), Beumer (37), and Campbell (6) have accepted this view rather grudgingly in recent papers. Beck and Wacker and Campbell in particular find it difficult to understand why it should be necessary for the cholesterol to be excreted and reabsorbed when it might be carried directly by the blood stream to the tissues for storage. They suggest that the cholesterol cycle may have something to do with the absorption of fat in an analogous manner to that of the bile acids. Hueck (28) believes that, if the process of reabsorption takes place at all, it is of small importance. It is indicated by the data obtained in the present work that the reabsorption of cholesterol is of considerable importance and that under some conditions at least, all of that entering the intestine with the bile may be reabsorbed.

An even more fundamental and general problem is that of determining whether cholesterol may be synthesized by the animal organism. The problem has been attacked by three types of experiment: autolysis and perfusion of organs; complete balance experiments with growing animals, involving analysis of intake, output, and body content; and, finally, balance experiments involving only intake and output. The first type has yielded no information of value and has been severely criticized by various writers. Experiments of the second type by Ellis and Gardner (38), Beumer

(5), and Thannhauser and Schaber (39) with incubated eggs; by Beumer (5) and Beumer and Lehmann (40) with growing pups; by Channon (41), Liang and Wacker (12), and Randles and Knudson (42) with growing rats; by Dezani (43) with growing mice; and by Fox and Gardner (44) with infants (computed in connection with balance experiments) leave little doubt that cholesterol certainly is not destroyed and may be synthesized if needed by growing animals. In all of the animal experiments except the old ones of Gardner and Lander (45) the increase in cholesterol of the body over that of the controls plus that excreted in the feces has been found to exceed the intake. Because of the possibility, brought out in the present paper, that fecal cholesterol may be synthesized by bacteria, the results of the foregoing experiments have been examined in order to determine what proportion of the "synthesized" cholesterol was represented by that excreted in the feces. If it were found that a large part were excreted in the feces, while the increase in body cholesterol was small, the case would not be nearly as clear in favor of cholesterol synthesis. It was noted that in experiments of Liang and Wacker about one-third, and in those of Channon over one-half of the "synthesized" cholesterol was represented by excretion through the feces (negative balance), but in the work of Beumer and Lehmann with pups the fecal cholesterol made up a very small portion of the increase over the intake. It seems quite certain that there is a synthesis of cholesterol by growing animals independent and in excess of any excretion through the feces.

The last type of experiment is the only one possible with adults and it has attracted considerable attention. The early work of Ellis and Gardner (46) with various animals, including man, led them to believe that there was no synthesis, as all the fecal cholesterol was accounted for by the food, despite the finding of negative balances with cats. Recently, however, Gardner and Fox (47), using improved methods with adult humans, found negative balances and now believe that some organ has the power to synthesize cholesterol. Balance experiments with infants by Gamble and Blackfan (48), Beck and Wacker (36), Beumer (37), and Fox and Gardner (44) led them to similar conclusions as did the work of Beumer (49) with pathological cases. The last named author finds it difficult to understand, however, why all the food cholesterol should be excreted while the body synthesizes cholesterol for its needs. Thannhauser (15) carried out cholesterol balance experiments over a period of 35 days with two adults and, though he obtained small negative balances during a 2 week period of low cholesterol feeding, he feels that the ability of the adult human to synthesize cholesterol, if it exists at all, is not very marked. The experiments recorded in this and the preceding paper show beyond question that the adult dog has the power to excrete sterol (of which a part is doubtless cholesterol) in fairly large amounts, though there is none in its food, and the view that it has the power to synthesize cholesterol would thus seem to be confirmed.

There is another factor, however, concerned in most of the experiments mentioned above, which has received very little notice. That is the possibility that fecal cholesterol comes from some other source than the bile

or food. Schmidt and Strasburger (50) mention the possibility that some may have its origin in the epithelium of the intestine but consider the "cholesterol-rich bile" as the principal source aside from the food. Beumer (37) also recognizes intestinal epithelium as a possible source of fecal cholesterol because of his finding of negative cholesterol balances in a case of occlusion of the common duct; but he nevertheless considers the bile as the principal source of the fecal cholesterol. Channon (41) apparently is the only writer who has recognized, in a criticism of the work of Gardner and Fox (47), that "bacterial synthesis of sterol in the large intestine may be the cause of the large excess of excretion over intake." Otherwise the old hypothesis of Bondzyński and Humnicki (51) that dog feces contain the unchanged cholesterol of bile is apparently generally accepted. Thus we find Gardner and Fox (47) and Beumer (37), the most active workers in the field, saying respectively, "The sterol excreted in the feces is derived partly from the food and partly from the bile;" and "The cholesterol of the feces is made up of the two components: unabsorbed food and bile cholesterol." It is indicated by the results of the present study that under some conditions the cholesterol of the feces comes from neither the food nor the bile but from some other source which may be secretion through the intestinal wall, desquamated epithelium, or bacteria, and some doubt is, therefore, thrown on the value of balance experiments designed to test the ability of the body to synthesize cholesterol.

EXPERIMENTAL.

Plan of Experiment, Etc.—The experiments consisted of the collection and analysis of the feces excreted by bile fistula[1] dogs receiving a lipid-free diet. The dogs employed had all been used in several experiments of the same nature with essentially the same diet while still normal and it was hoped that a comparison of the results obtained before and after operation would throw much light on the relationship between the bile and the fecal lipids. The experiments were continued over long periods of time (up to 5 weeks) and the feces were collected in weekly periods as described in previous papers of this series (1, 2).

There was at intervals some constipation and from time to time warm water enemas were given. It was the usual practice to put the animal on the experimental diet for 2 or 3 days and then to give an enema on the day of charcoal feeding in order to wash out all residual fecal matter. Enemas were frequently given on the 2nd day after feeding the charcoal marker, which marked one

[1] The term "bile fistula" is used in this paper to signify that the bile is completely excluded from the intestine.

weekly period from the next, because it was found easy to obtain very accurate demarcation by this means. That the enema itself had no effect on the results was shown by a number of weekly experiments scattered throughout the work (Experiments 10B,[2] 11A, 11B, 12, 13A, 13B, 13E) where it was not found necessary to use an enema as the excretion was regular and the demarcation good. The results of these experiments agreed with those where the enema was used.

The stools were at all times hard and well formed and light gray in color, indicating the absence of bile from the intestine.

There was a gradual small loss of weight in most of the series, though in Series 9 and Series 13 the weight was kept almost constant with a slight gain near the middle of the series in each case and a drop to the starting value at the end. In the fasting experiment there was of course a large decrease in weight and in the last week of Series 11 (Experiment 11C) Dog 24–10 ate very poorly and lost considerable weight. The same is true to a lesser degree in the case of Dog 24–9 in Experiment 14D. No effect of these weight fluctuations was observed.

Two controls were used, the first being the data obtained with the same dogs while still normal and the second being a similar series (Series 6, Table I) with normal dogs, in which exactly the same diet and technique were used and which were carried out simultaneously with Series 9 and 10.

Diet.—It was clearly recognized that the diet must be very carefully controlled with respect to its lipid content because of the well known poor absorption of these substances by bile fistula dogs. The diet was made up in general according to the recommendations of Cowgill (52). The constituents and amounts fed per kilo per day were as follows: 6.4 gm. of casein, 13.6 gm. of sugar, 0.4 gm. of bone ash, 0.28 gm. of salt mixture (Cowgill), and 0.6 gm. of Vitavose.[3] These constituents were mixed in large amounts

[2] In this paper the analysis of the fecal output of a dog over a period of 1 week is called an "experiment." Such experiments are designated by letters. A number of consecutive weekly experiments is called a "series" and the series are designated by numbers. Thus Experiment 10B means the experiment covering the 2nd week of Series 10.

[3] The Vitavose was supplied through the courtesy of Dr. Charles Hoffman of the Ward Baking Company.

in the proportions indicated and each day the required amount for each dog was weighed out and thoroughly mixed with an amount of water equal to the body weight in kilos multiplied by 1.75 cc. During all the experiments except the last half of Experiments 13B, 13C, 13D, 13E, and all of Series 14 an amount of meat extract equal to the body weight in kilos multiplied by 0.5 gm. was dissolved in the water. Three constituents of the diet, the casein, Vitavose, and meat extract, might contain lipids and these were treated and examined as follows.

Casein.—The casein was extracted in a modified Clarke apparatus as described in the preceding paper of the series (1). A discussion of the difficulties met with in completely extracting the lipids is given there (p. 360). It was finally found possible by repeated extractions, between which the casein was finely ground, and which usually totalled about 50 hours, to obtain material containing 0 to 0.03 per cent total lipids. The method of analysis consisted in digesting 10 gm. portions for 24 hours on the steam bath with strong alkali, acidifying the sulfuric acid, extracting with ether, and finally extracting the ether residue with petroleum ether. Material showing a lipid content of 0.02 per cent by this method showed none at all on extraction with petroleum ether in a Soxhlet apparatus. In most of the work the casein used contained 0 to 0.01 per cent, though in one instance it was necessary to use material containing 0.05 per cent. For some reason the extraction procedure was not as efficient as usual in this case and since no other was ready casein containing 0.05 per cent total lipids had to be used and formed about half of that employed for 4 days in Experiment 6B and 2 days in Experiments 9D, 10B, and 10C. No difference in the results, which might be ascribed to this small amount of lipid, was noted.

Vitavose.—This substance was used according to the recommendation of Cowgill as a source of vitamin B in order to stimulate appetite. It was analyzed by the same procedure employed with the casein except for the removal of volatile acids by triple distillation to small volume after acidification. Six determinations gave an average value of 0.08 per cent lipid material with maximum and minimim values of 0.12 and 0.04 per cent. One sample was extracted in a Soxhlet apparatus with petroleum ether for 5 days and showed a lipid content of 0.03 per cent.

Meat Extract.—The method of analysis of this substance was the same as used for the Vitavose. Four determinations gave an average value of 0.075 per cent with maximum and minimum values of 0.11 and 0.04. The material from all the determinations was combined and separated into unsaponifiable and fatty acid fractions. The ratio of the former to the latter was found to be 0.82. By the method of Soxhlet extraction a value of 0.01 per cent was obtained. That the meat extract had no effect on the results is further shown by the experiments in which it was omitted (see above).

It seems quite definite from the above analyses that the amounts of lipids present in the diet were much too small to have had any effect on the results.

Operative Technique,[4] *Condition of Animals, Etc.*—Five dogs were used in all. The first was a bile fistula animal which chanced to be available in the laboratory and it was used principally to try out the diet and technique. Since no control is available, the results from this dog are not included, although in general they agreed with the others.

The operation on the second dog, No. 23–2, consisted in fastening a cannula in the gall bladder by means of a purse string suture and cutting the common duct between double ligatures. A long rubber tube with glass bend was used according to the directions of Rous and McMaster (53), but it was attached to a metal cannula fastened in the abdominal wall and no attempt was made to exclude bacteria from the open end of the cannula or the surrounding area. Unfortunately, through an unforeseen circumstance, it was impossible to use this dog in an experiment for a period of over 2 months from the date of the operation. During this time it lost considerable weight and the area about the cannula became rather extensively infected. It was also necessary from time to time to clean out the bile tract by lavage, under alternate small increases and decreases of pressure, with dilute sodium bicarbo-

[4] The operation on Dog 23–2 was performed by Dr. G. H. Whipple assisted by Dr. H. P. Smith, both of the Department of Pathology; on Dogs 24–21 and 24–10 by Dr. H. P. Smith assisted by Dr. Robert Angevine of this department; and on Dog 24–9 by Dr. J. J. Morton of the Department of Surgery assisted by Dr. H. P. Smith. The thanks of the author are due to these men for making this investigation possible.

nate solution. During the first experiment (Experiment 8) with this dog, however, the bile flowed quite freely and the animal appeared active and healthy and ate its food fairly well. During this experiment it was prevented from licking the bile by means of a muzzle covered with a fine wire cloth. The dog was constantly watched while the muzzle was off for purposes of eating and drinking.

Considerable trouble was experienced during the 1st week of the second series with this dog (Series 9) due to stoppage of the bile tract. By repeated treatment with bicarbonate solution the flow was finally started and was free during the remainder of the series. The bile was abnormally light in color throughout though it became darker toward the end. There was no evidence of jaundice after the 1st week and little then. In this series the bile was collected in a rubber balloon placed in a metal box, which was held in position by a canvas binder. During this series the dog was very active, ate its diet completely and greedily every day, and appeared perfectly normal. Though there was doubtless considerable infection in and about the bile tract, it is not thought that it had any appreciable effect on the results obtained, both in view of the normal appearance of the dog and the agreement of the results with those from other animals with sterile fistulous tracts. The dog died about 3½ weeks after the conclusion of Series 9 of general peritonitis. It was found at autopsy that there were many adhesions and much infection about the tube but the cannula was still in place.

The operation on the third dog, No. 24–21, was essentially that described by Rous and McMaster (53), the cannula being inserted in the common duct and the gall bladder removed. The bile was collected in a sterile balloon and the area about the tube was kept sterile. During the first 2 weeks the balloon was disconnected and emptied daily with sterile technique, but later a side tube was added and the bile emptied as described by Rous and McMaster. The bile was frequently cultured[5] and was found sterile at all times.

The experiments with this animal (Series 10) were started as

[5] The examinations of the bile were carried out by Dr. Stanhope Bayne-Jones of the Department of Bacteriology, to whom the thanks of the author are due.

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soon as it had fully recovered from the operation. The dog ate well throughout, appeared normal, and the experiments ran very smoothly. It died about 2 weeks after the conclusion of the experiments of general peritonitis caused by intestinal obstruction. The cannula was found in place at autopsy.

The operation on Dog 24–10 was the same as in the case of Dog 24–21. Series 11 was started with this dog as soon as it had recovered from the operation and went smoothly except for the dog's refusal to eat during the last week. At the conclusion of this series it was placed on the regular laboratory diet, consisting principally of bread and liver, for 3 weeks and then, though it had gained little weight, was fasted for a week (Experiment 12), receiving only agar jell containing 2.5 gm. of bone ash per day to stimulate intestinal activity. About 2 weeks later Series 13 was started with the standard lipid-free diet. This was the longest and best series obtained. The dog ate its food completely throughout, maintained weight, and was apparently in good health at all times. The bile was frequently cultured[5] and with one exception, when it became infected during removal from the balloon, was always sterile. At the conclusion of the series the dog was killed and at autopsy was found normal in all respects with no signs of infection and with only a few minor adhesions about the pancreas. The cannula was in place and the tube was surrounded by a sheath as described by Rous and McMaster.

The operation on the last dog, No. 24–9, consisted of cholecystnephrostomy, as described by Kapsinow, Engle, and Harvey (54), performed in two stages. The recovery was good, bile appeared in the urine at once in large amounts, and the feces became clay-colored. The dog was kept in a cage with a screen bottom so there could be no contamination of the feces by the bile-containing urine.

This dog refused to eat the experimental diet at the start of Series 14 and finally feces were collected over a period of approximately 5 days (Experiment 14A), during which the animal was almost completely fasting. There was some evidence of jaundice during this period. On the 6th day it commenced to eat and continued to for 2 weeks. During the last week (Experiment 14D) it again refused to eat well and left over half its food. This dog is still alive and is being used for another purpose, so no autopsy is

available. The precautions taken at operation together with the clay-colored stools and the agreement of the results with those from the other dogs make it extremely improbable that any bile was reaching the intestine during the experiments.

Analytical Methods.—The procedure employed was essentially the same as described in previous papers (1, 2). It was found possible by the use of reduced pressure to distil the entire acidified mixture of fecal digest on the steam bath without filtration, and this technique was used in Experiment 10D and those following. There was little frothing or bumping and it was possible to distil to dryness, a procedure which was repeated three times in each case in order to remove the volatile acids, which were discarded. The entire residue in the flask was then thoroughly mixed with ether and filtered, this being repeated a number of times. The ether solution was washed with water and the analysis carried on as before. The advantages of the change in procedure described above are the entire avoidance of the unpleasant odors obtained by the old method and a considerable shortening in the work, only one extraction being required.

As indicated in the introduction it was deemed advisable to lay particular emphasis on the application of this work to certain problems of cholesterol metabolism. It seemed desirable, therefore, to obtain as much information as possible concerning the cholesterol content of the excreted lipids. There are but two general methods available for the determination of cholesterol: the gravimetric, involving precipitation with digitonin, and the colorimetric. The first, considered by many to be the more accurate, could not be used because no digitonin was available. The second, as applied to human fecal sterols, has been very severely criticized by Gardner and Williams (55) because of the difficulty in matching the colors obtained and because of the occurrence of feeble colors with other amorphous sterols. However, since it was the only one available, it was determined to use the colorimetric method in the hope that some information of a comparative nature at least might be obtained, and all the unsaponifiable fractions available from the lipid-free experiments with both normal and bile fistula dogs were analyzed by the method of Bloor, Pelkan, and Allen (56).

It was found with considerable surprise that in the majority of

TABLE I.

Lipid-Free Series 6 (Normal Dogs).

Dog No.	Weight of dog.	Total lipids. A	Unsaponifiable.		Non-volatile fatty acids.			Solid fatty acids.				Liquid fatty acids.			Ratio of liquid to solid fatty acids. $\frac{E}{D}$
			Weight. B	In total lipids. $\frac{100B}{A}$	Weight. C	In total lipids. $\frac{100C}{A}$	Melting point.	Weight. D	In total lipids. $\frac{100D}{A}$	In non-volatile fatty acids. $\frac{100D}{C}$	Melting point.	Weight. E	In total lipids. $\frac{100E}{A}$	In non-volatile fatty acids. $\frac{100E}{C}$	
Experiment 6A.															
	kg.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	
24–9	8.15	1.441	0.542	37.6	0.754	52.3	36	0.298	20.7	39.6	51	0.363	25.2	48.2	1.22
24–10	8.00	2.124	0.556	26.2	1.354	63.8	37	0.489	23.0	36.1	49	0.721	34.0	53.3	1.48
24–11	9.95	2.214	0.721	32.6	1.262	57.2	34	0.466	21.1	37.0	47	0.690	31.2	54.6	1.48
Average.....	8.70	1.926	0.606	32.1	1.123	57.8	36	0.417	21.6	37.6	49	0.591	30.1	52.0	1.39
Experiment 6B.															
24–9	8.15	2.309	0.893	38.6	1.212	52.5	37	0.480	20.8	39.6	47	0.572	24.8	47.2	1.19
24–10	8.15	2.464	0.631	25.6	1.717	69.5	36	0.596	24.2	34.8	47	0.824	33.4	48.0	1.38
24–11	10.50	1.734	0.543	31.3	1.078	62.2	35	0.306	17.7	28.4	48	0.551	31.8	51.3	1.80
Average.....	8.93	2.169	0.689	31.8	1.336	61.4	36	0.461	20.9	34.3	47.3	0.649	30.0	48.8	1.46
Experiment 6C.															
24–9	7.97	2.179	0.684	31.4	1.209	55.5	35	0.464	21.3	38.4	48	0.617	28.4	51.1	1.33
24–10	7.75	1.701	0.443	26.0	1.069	62.8	27	0.394	23.2	37.0	48	0.571	33.6	53.5	1.45
24–11	10.30	2.070	0.602	29.1	1.229	59.4	25	0.429	20.7	34.9	49	0.650	31.4	53.0	1.52
Average.....	8.67	1.983	0.576	28.8	1.169	59.2	29	0.429	21.7	36.8	48.7	0.612	31.1	52.5	1.43

cases the color from the fecal unsaponifiable material was of exactly or very nearly the same hue as from the pure cholesterol standard, and that the two could, therefore, be closely matched. In a few cases there was a considerable difference in hue, making the colors difficult to match. In these cases it was found possible to compare the solutions by the aid of a red filter such as is used in this laboratory rather infrequently in analyses of blood cholesterol where the colors do not match well. The same filter was tried in several of the determinations where good readings had been obtained without it and essentially the same values were found. In comparing these findings with those of Gardner and Williams it must be remembered that they were working with human feces while the data presented here were obtained with dog feces. It may be that the interfering substances which they found are present in smaller amount in the latter than in the former. It is recognized that the values obtained probably do not represent the exact amounts of cholesterol present but it is thought that they approximate the true percentages and are certainly of value in comparing the excretion by normal and bile fistula dogs.

Explanation of Tables.—Table I contains the data obtained in Series 6 with normal dogs on the strictly lipid-free diet employed in the bile fistula work. These experiments serve not only as a control for the results obtained with the bile fistula dogs, but also as a control for the experiments with normal dogs reported in the preceding paper (see foot-note on p. 361 (1)).

In Table II are collected the data obtained with bile fistula animals. The results of all the experiments carried out with lipid-free diets while the dogs were still normal have been averaged in the case of each of the animals and are included in the table for comparison. One experiment, No. 12, is a fasting experiment. Through accident small portions of the ether extracts of the feces of Experiments 14A and 14B were intermixed and the data are, therefore, of little value. However, the experiments of the last weeks of a series are of much more value than the first, as the possible influence of previous diet becomes less and less, and fortunately the experiments of the last 2 weeks of this series ran very smoothly and gave results in agreement with those from the other animals.

In Table III are given the averages of certain experiments with

TABLE II.

Composition of Fecal Lipids Excreted by Bile Fistula Dogs on a Lipid-Free Diet.

Dog No.	Experiment No.	Weight of dog.	Total lipids. A	Unsaponifiable.		Non-volatile fatty acids.			Solid fatty acids.				Liquid fatty acids.			Ratio of liquid to solid fatty acids. $\frac{E}{D}$
				Weight. B	In total lipids. $\frac{100B}{A}$	Weight. C	In total lipids. $\frac{100C}{A}$	Melting point.	Weight. D	In total lipids. $\frac{100D}{A}$	In non-volatile fatty acids. $\frac{100D}{C}$	Melting point.	Weight. E	In total lipids. $\frac{100E}{A}$	In non-volatile fatty acids. $\frac{100E}{C}$	
		kg.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	
23–2	Average of 6 normal experiments.	12.3	1.818	0.631	35.5	1.099	60.7	37	0.335	18.4	30.3	49	0.650	35.2	58.1	2.07
23–2	8	9.4	5.475	2.066	37.8	3.033	55.4	39	1.311	24.0	43.3	55.5	1.327	24.2	43.8	1.01
	9A	9.15	6.268	2.366	37.8	3.612	57.6	39	1.381	22.1	38.3	51	1.714	27.4	47.5	1.24
	9B	9.40	7.606	5.788	76.0	1.472	19.4	35	0.318	4.2	21.6	50	0.895	11.8	60.8	2.82
	9C	9.10	5.568	1.910	34.3	3.020	54.3	37	1.046	18.8	34.6	49	1.457	26.2	48.2	1.39
	9D	9.10	8.663	2.733	31.5	5.677	65.5	40	1.862	21.5	32.8	55	2.836	32.7	49.9	1.52
24–21	Average of 6 normal experiments.	8.4	1.696	0.532	3.70	1.067	62.9	37.6	0.334	19.6	31.0	49	0.578	34.9	54.9	1.61
24–21	10A	9.8	4.854	1.446	29.7	3.022	62.2	42	1.293	26.6	42.8	52	1.392	28.7	46.0	1.08
	10B	9.7	7.747	2.934	37.9	4.727	61.1		1.901	24.6	40.2	56	2.301	29.8	48.7	1.21
	10C	9.5	6.877	1.747	25.4	4.115	60.0	40	1.713	25.0	41.6	55	2.038	29.6	49.5	1.19
	10D	9.2	4.232	1.321	31.3	2.574	61.0	43	1.003	23.8	39.0	56	1.131	26.8	44.0	1.13

	10C	9.5	6.877	1.747	25.4	4.115	60.0	40	1.713	25.0	41.6	55	2.038	29.6	49.5	1.19
	10D	9.2	4.232	1.321	31.3	2.574	61.0	43	1.003	23.8	39.0	56	1.131	26.8	44.0	1.13
24–10	Average of 13 normal experiments.	7.4	1.875	0.607	32.6	1.116	61.8	36.3	0.354	18.7	29.7	48	0.653	35.0	56.7	2.03
24–10	11A	7.75	5.583	1.739	31.2	3.634	65.0	50	1.811	32.5	50.0	56.5	1.570	28.2	43.3	0.87
	11B	7.75	3.261	0.922	28.2	2.187	67.1	47	0.922	28.2	42.1	57	0.991	30.4	45.3	1.07
	11C	7.8	3.076	0.901	29.3	2.050	66.7	47.5	0.996	32.4	48.6	57	0.966	31.4	46.7	0.97
	12 Fasting.	**7.1**	**3.131**	**1.100**	**35.2**	**1.778**	**56.8**	**39**	**1.005**	**32.1**	**56.6**	**58**	**0.640**	**20.4**	**36.0**	**0.64**
24–10	13A	5.9	4.567	1.242	27.2	3.083	67.5	39	1.556	34.0	50.4	56.5	1.269	27.8	41.0	0.82
	13B	6.3	5.199	1.524	29.4	3.414	65.8	37	1.426	27.4	41.8	56.5	1.637	31.5	48.0	1.15
	13C	6.4	4.619	1.291	28.0	3.115	67.5	42	1.403	30.4	45.1	55	1.470	31.8	47.3	1.05
	13D	6.15	2.651	0.938	35.4	1.575	59.5	44	0.662	25.0	42.0	56.5	0.652	24.6	41.5	0.99
	13E	5.8	3.556	1.056	29.7	2.657	74.6	45	1.254	35.3	47.3	55.5	1.015	28.6	38.3	0.81
24–9	Average of 14 normal experiments.	6.9	2.031	0.808	39.8	1.076	53.1	37.3	0.367	18.2	33.9	48.6	0.579	28.9	54.8	1.68
24–9	14A*	8.3	5.411	2.338	43.1	2.626	48.5	43	1.403	25.9	53.5	48	1.166	21.5	44.3	0.83
	14B*	7.5	3.698	1.316	35.6	2.125	57.6	42	0.977	26.4	46.0	49	0.908	24.6	42.7	0.93
	14C	7.3	4.350	1.280	29.4	2.818	64.8	41.5	1.027	23.6	36.5	49.5	1.292	29.7	46.0	1.26
	14D	6.93	4.916	1.527	31.0	2.988	60.8	41	1.444	29.4	48.4	49.5	1.328	27.0	44.4	0.92

*Data only approximate due to partial mixing (see text). Experiment 14A covers about 5 days during which the dog ate very little.

TABLE III.

Grand Averages of Experiments with Lipid-Free Diet.

Experiments included.	Weight of dog.	Total lipids. A	Unsaponifiable.		Non-volatile fatty acids.			Solid fatty acids.				Liquid fatty acids.			Ratio of liquid to solid fatty acids. $\frac{E}{D}$
			Weight. B	In total lipids. $\frac{100B}{A}$	Weight. C	In total lipids. $\frac{100C}{A}$	Melting point.	Weight. D	In total lipids. $\frac{100D}{A}$	In non-volatile fatty acids. $\frac{100D}{C}$	Melting point.	Weight. E	In total lipids. $\frac{100E}{A}$	In non-volatile fatty acids. $\frac{100E}{C}$	
	kg.	*gm.*	*gm.*	*per cent*	*gm.*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	*°C.*	*gm.*	*per cent*	*per cent*	
All experiments with normal dogs except Series 6.	8.5	1.823	0.685	37.9	1.045	57.5	37.0	0.311	17.3	29.9	49.0	0.577	32.8	57.3	2.02
Series 3 with normal dogs.	8.75	1.779	0.584	33.1	1.104	61.9	37.0	0.362	21.1	34.2	48.1	0.578	34.3	55.4	1.65
Series 6 with normal dogs.	8.76	2.026	0.623	30.9	1.209	59.5	33.7	0.436	21.4	36.2	48.3	0.617	30.4	51.1	1.43
All experiments with bile fistula dogs except Experiments 9B, 12, 14A, 14B.	7.94	4.905	1.608	31.4	3.183	63.1	42	1.303	26.9	41.9	54.4	1.466	28.7	45.5	1.09

normal and bile fistula dogs in order to facilitate the comparison with respect to composition.

TABLE IV.

Cholesterol Determinations.

Experiment.	"Cholesterol."
	per cent
Lipid-free 1, normal.*	49.0
" 2A, normal	39.7
" 2B " *	31.2
" 2D " *	67.5
" Series 3	41.8
" 4, normal	54.6
" 5 "	47.2
" 6A "	52.4
" 6B " *	47.6
" 6C "	54.2
" 8 (bile fistula)	24.6
" 9A " "	38.9
" 9B " " *	18.0
" 9C " "	34.0
" 9D " "	40.6
" 10A " "	42.5
" 10B " "	29.8
" 10C " "	46.4
" 10D " "	37.6
" 11A " "	38.3
" 11B " "	32.8
" 11C " "	46.0
Fasting 12 " "	28.8
Lipid-free 13A " "	39.7
" 13B " "	37.6
" 13C " "	38.0
" 13D " "	34.0
" 13E " "	48.3
" 14B " " *	39.0
" 14C " "	48.0
" 14D " " *	36.0

* Difficult to match.

Table IV contains the results of the cholesterol determinations. Experiments where it was difficult to compare colors and where a red filter was used are indicated by asterisks. The term "cho-

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lesterol" has been placed in quotation marks to indicate that it is only approximate and may be influenced by other sterols. It should be noted that the determinations are not all of equal importance since in some cases, particularly with the normal dogs, the unsaponifiable fractions from several animals (in Series 3 from all five experiments) had been combined and were analyzed as a mixture.

DISCUSSION.

The primary problem of this investigation was that of determining whether the lipids excreted by normal dogs on lipid-free diets reach the intestine by way of the bile or come from some other source. If, when bile is excluded from the intestine, the excretion continues as before in amount and composition, it appears logical to draw the conclusion that the bile is not the source of fecal lipids. The discussion will, therefore, be principally concerned with a comparison of the excretions of normal and bile fistula animals.

The data at hand show beyond question, not only that the excretion does continue after operation, but that it continues in considerably larger amounts than in normal animals. Can this increased excretion be correlated with the hypothesis that the lipids excreted by bile fistula dogs come from the same source (not the bile) as those excreted by normal dogs? The possible criticism must be recognized that the excretion of the bile fistula dog is a different thing and comes from a different source than does that of the normal as witnessed by the larger amount excreted by the former. Such a criticism can best be answered by a comparison of the two excretions with respect to composition (see below) and by pointing out that it is entirely possible to explain the increased excretion without giving up the hypothesis that it comes from the same source as in the normal dogs. In fact three such explanations have been suggested.

According to the first of these there is a secretion of lipids throughout the length of the intestine which may have a useful purpose or simply represent a "leakage" from the blood. In the normal dog this secretion is reabsorbed to a large extent but in the dog from the intestine of which the bile is excluded such reabsorption is difficult or impossible. This view coincides to some extent with that of Niemann (57) who observed a case of congenital occlu-

sion of the bile duct. He studied the infant during the 9 months of its life and found during the last period of observation a negative lipid balance of about 5 gm. per day. He advances the theory that the excretion accompanies the transport of large quantities of fat from the tissues and apparently thinks that there is a leakage into the intestine dependent in quantity on the amount of fat being carried by the blood. He thinks that since this fat cannot be reabsorbed it appears in the stool. He also thinks it possible that normally in conditions of undernourishment large amounts of fat appear in the intestine but in the presence of bile are reabsorbed.

A second theory explains the excretion as due to desquamation of intestinal epithelium. As in the first hypothesis the lipids from the upper portions are thought to be absorbed by the normal dog but excreted by the bile fistula dog. It is evidently very difficult to differentiate between a desquamation of this sort and a secretion, and the two theories are, therefore, very similar.

The third explanation postulates bacteria as the source of the increased excretion. It has long been believed that the bile acids are disinfecting agents (Röhmann (58)) and that there is increased bacterial action when the bile is excluded from the intestine. It has been mentioned before that Channon (41) has recognized the possibility that bacterial synthesis may explain negative lipid balances. However, as pointed out in the preceding paper, rough calculations on the basis of such few unsatisfactory analyses of bacterial lipids as are available, indicate that but a small portion of the excretion of normals may be thus accounted for; and it is rather difficult to believe that such large amounts as have been found in the feces of bile fistula dogs are formed by bacteria. Still the possibility cannot be ruled out at present. Further work is being planned in the hope of obtaining more information concerning the lipid content of intestinal bacteria.

If the fecal lipids excreted by bile fistula dogs had the same characteristics and composition as those excreted by normal dogs, it would be strong evidence for the view that both come from the same source. Casual examination reveals several points of close similarity in the excretions under the two conditions. As in the normals there is a large variation in the total excretion of each dog from week to week. Also the percentage composition, as far as the four fractions determined go, is quite constant throughout in

agreement with the results of the work with normals. In only one case (Experiment 9B) was there any marked variation from the average. In this experiment an abnormally large amount of unsaponifiable material was found along with an abnormally small amount of fatty acids. It was thought that the saponification might have been incomplete and several attempts were made to break down the unsaponifiable fraction further by means of alcoholic potassium hydroxide, but without success. Why there should have been this single abnormal case is not understood.

The excretion continues over periods of time up to 5 weeks in length with no apparent change either in total amount or percentage composition. In fact the largest excretion was obtained in the last week of a series (Experiment 9D). As far as the constancy of percentage composition goes, this finding is in complete agreement with the results from normal dogs; but in the previous paper the conclusion was drawn, based largely on the results from Series 2, that there was a small diminution in total excretion toward the end of a series. Series 2 did not run smoothly, however, due to the pathological condition of the dogs, and the diminution was not nearly as marked in Series 3 where there was, in fact, a decided rise during the 4th week. In Series 6, which ran very smoothly throughout, there was no evidence of such a diminution and it is felt now that, if there is any such regular diminution in the excretion of normal dogs, it is very small. Certainly there is no great difference between the results with normal and bile fistula dogs as far as this point is concerned.

There apparently is a rough proportion between the average total excretion of the bile fistula dogs and their body weight. The variations from week to week are so great, however, that it is difficult to say whether this relationship is real or apparent. No such proportion could be shown in the case of the normal dogs. Another possible point of dissimilarity is to be found in the fasting experiment, where the excretion was as large as in several of the experiments with the lipid-free diet. In the case of normal fasting dogs definitely smaller amounts were excreted than with the lipid-free diets. It is possible to explain this apparent discrepancy by the assumption that the normal fasting dog, in his effort to conserve all his resources, reabsorbs a larger amount of secreted lipids than he does on an ample diet. The bile fistula

dog on the other hand, is unable to reabsorb lipids from the intestine under any conditions. In agreement with the fasting experiment no effect of decrease in weight because of poor eating was observed. The same was found in the work with normals though it was not understood why there should not have been a decrease in the excretion as in the fasting experiments. Taking it all in all, as far as the above general considerations go, there is a marked similarity in the excretion before and after exclusion of the bile from the intestine. Such apparent differences as have been noted are of minor importance and for the most part may be explained on the assumption that the fecal lipids of bile fistula dogs come from the same source as those of normal dogs.

Before comparison can be made with respect to percentage composition it is necessary to say a word concerning the composition of the basal lipid excretion of normal dogs. Because of the discrepancy between Series 2 and Series 3, discussed in the preceding paper, it was difficult to determine what that composition was and it was decided, temporarily at least, to strike a rough average between the two series. Another series with normals (Series 6, Table I) is now available and in order to facilitate comparison the data from this series have been averaged and are presented in Table III along with the average values of certain other experiments. The averages of all experiments with normal dogs on lipid-free diets except Series 6 are given in the first row. These are the values which were taken as representing the normal basal excretion in the preceding paper. It will be noted that there is a decided, though not large, difference between these values and the averages of Series 6. The ratios of unsaponifiable material to fatty acids and of liquid to solid acids are definitely smaller in the latter. The values from Series 3 have also been separately averaged. A comparison between these figures and those from Series 6 shows fairly close agreement. The ratios mentioned above are a little larger in Series 3 than in Series 6, it is true, but the agreement is much closer than between either of these series and Series 2, to which the high ratios in the grand average (first row of table) are largely due. It must be remembered, as mentioned above, that the results of Series 2 are open to question because of the pathological condition of the animals, while both Series 3 and Series 6 ran very smoothly. It is felt that the results of Series 6

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in particular deserve special consideration, despite the smaller number of dogs and experiments, since no trouble of any kind was experienced and the dogs ate well and were healthy throughout. It may be that the slight differences in composition with respect to Series 3 are due to improved analytical technique obtained with practice. It is not thought that the use of a strictly lipid-free diet, rather than one containing small amounts of lipids in the casein, made any difference in the results, since the total excretion was a little larger with the strictly lipid-free diet. From these considerations it seems best to take values intermediate between Series 3 and Series 6 as representing the normal composition of the fecal lipids excreted by normal dogs on a lipid-free diet.

We are now in a position to compare the average composition of the lipids excreted by bile fistula dogs with these normal values. It is noted at once that the percentages of unsaponifiable material and fatty acids are almost exactly the same as those adopted as normal. It is just as apparent, however, that the melting points of the total fatty acid fractions are consistently high and, as might be expected from this fact, the ratios of liquid to solid acids are definitely low in the case of the bile fistula dogs. It may be that there is a higher selective reabsorption of solid than of liquid acids from the secreted lipids in the normal dog, from which one might conclude that the bile is of special value in the absorption of solid acids. Another and perhaps better explanation is that the lipids, either secreted or from desquamated epithelium, in the upper portion of the intestine, where they normally are reabsorbed, contain a higher proportion of solid acids than those secreted in the lower part; or, if it be assumed that the excretion is due to bacteria, it would rather be expected that there would be a difference in the types of bacteria with probable difference in composition of lipids in bile fistula dogs. At any rate, whatever the explanation may be, the difference is comparatively small, and in view of the very close similarity in the proportion of unsaponifiable and fatty acid fractions, it seems most probable that the excretion comes from the same source before and after the exclusion of the bile from the intestine.

The results are of interest in view of the possibility, expressed in the preceding paper, that the excretion may have to do with the removal of waste sterols, the function of the fatty acids being that

of holding the sterols in solution. The high ratio of liquid to solid acids and low total acids in the normal fasting experiments were thought to be of possible significance as expressing the ability of the animal to accomplish its purpose with less wastage of usable acids. If the purpose of the excretion is that of removing waste sterols from the body through the intestinal wall, one would expect that the same proportion of total acids of the same composition would be required whether the excretion of sterol were large or small. A change in the ratio of liquid to solid acids would not be expected unless there were a change in the ratio of unsaponifiable material to total acids. In other words one might expect, if the above theory were tenable, that a decrease in the proportion of total acids would be accompanied by an increase in the proportion of liquid acids. A tendency in this direction indeed is shown apparently by the values in Table III, a decreasing proportion of unsaponifiable material being accompanied by a decreasing ratio of liquid to solid acids. It seems to go too far, however, in the case of the bile fistula experiments and we see a marked decrease in the latter ratio without an accompanying decrease in the proportion of unsaponifiable material. The high ratio of liquid to solid acids in Experiment 9B, where there was an abnormally high proportion of unsaponifiable material, seems to be in accord with the theory; but, when the ratios of liquid to solid acids in the other experiments are plotted against the percentages of unsaponifiable material, no relationship at all is seen. The very low ratio in the fasting experiment is also apparently in direct contradiction to the results of the fasting experiments with normal dogs. The theory that the fatty acids of the fecal lipids are present in order to hold the sterols in solution receives little support from the results of this investigation.

Time was not available for the detailed analysis of the fatty acid fractions such as was carried out in the work with normal animals. The melting points of the solid acid fractions in most cases are close to the theoretical for a mixture of equal parts of palmitic and stearic acids. Apparently there was better separation made possible by the larger amounts of material available, the lower melting points in the experiments with normals being due to the presence of small amounts of liquid acids.

Information of a comparative nature is available concerning the

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composition of the unsaponifiable fractions (Table IV). The "cholesterol" content of the fractions excreted by normal dogs seems to be somewhat higher on the average than of those excreted by bile fistula dogs. The apparent average of values from normal dogs is 48.5 while that from the bile fistula dogs (omitting Experiment 9B) is 38.0. It must be remembered, however, that the percentages from the normal animals are by no means of equal value since they represent varying numbers of experiments. The analysis labelled Series 3 is of the greatest importance because the sterol fractions from the greatest number of experiments were combined in this determination. It is to be noted that the value obtained is very close to the average of the bile fistula experiments. Gardner and Fox (47) in twenty-six determinations found an average of 59.5 per cent of digitonin-precipitable sterols but this was in human feces. There was considerably more variation in the results of the analyses of the material from normal dogs than of that from the bile fistula animals. This finding may be related to the fact that in some of the ether solutions, which had been stored for considerable periods, varying amounts of a white, insoluble substance had formed. Time was not available for an investigation of its nature. The abnormally low percentage in the case of Experiment 9B is of interest in connection with the large amount of unsaponifiable material excreted in that experiment. The appearance of the color was the same from normal and bile fistula dogs and it is not thought that the difference found, if it is a real difference at all, is sufficient to lead to the conclusion that the sterols come from different sources in normal and bile fistula dogs.

Considering all the foregoing evidence as a whole, it seems more reasonable to conclude that the excretion comes from the same source in bile fistula as in normal dogs, rather than to assume that in the latter it has its source in the bile while in the former it is a different excretion and is formed in some other way. In general the excretions under the two conditions have been found to be very like and such differences in characteristics and composition as have been discovered are small and may be explained as due to the different condition of the intestine in the absence of bile.

This investigation was undertaken particularly because of its application to certain problems of cholesterol metabolism, as discussed in the introduction. If it is accepted that the fecal

lipids come from the same source in bile fistula as in normal dogs, it follows at once that the basal lipid excretion of a normal dog, including the cholesterol, does not come from the bile. This means that the view of a great many investigators, that the bile is the normal pathway of cholesterol excretion, must be modified. The present investigation of course presents no evidence to show that excess cholesterol from the food is not absorbed and reexcreted by way of the bile. However, the evidence of other investigations discussed in the introduction does not support such a view and it seems quite definite from the present results that when the animal is receiving no cholesterol none is removed from the body by the bile. It also follows that the cholesterol present in the bile must be completely reabsorbed under the conditions of these experiments in some such fashion as was postulated years ago by Gardner and coworkers. Finally, the results raise a question concerning balance experiments designed to test the ability of the body to synthesize cholesterol. Though negative balances have been obtained, seemingly confirming the view that cholesterol may be synthesized, the possibility that bacteria are responsible must be eliminated before cholesterol synthesis by the adult animal organism may be considered established. Even if the source of the fecal lipids is found to be desquamation of intestinal epithelium or a secretion of lipids into the intestine for the purpose of lubrication, it is not necessarily proved that there is a synthesis of cholesterol. It is possible that the relatively small amounts of cholesterol removed from the body by this means are replaced from body stores.

CONCLUSION.

A comparison of the lipids excreted by dogs before and after exclusion of the bile from the intestine reveals but one outstanding difference: a considerably larger amount is excreted after exclusion than before. Minor differences, of which the most obvious is a smaller ratio of liquid to solid acids after operation, are noted in the composition; but these differences may be correlated with the hypothesis that the fecal lipids come from the same source in bile fistula and normal dogs. The increased excretion by the former may also be correlated with the same theory by the assumption that throughout the length of the intestine there is a secretion

of lipids, which are largely reabsorbed by normal dogs but which escape absorption in the absence of bile. It is strongly indicated by the general similarity in composition of the lipids excreted by normal and bile fistula dogs that in each case they come from the same source, which, of course, cannot be the bile. These results are not in agreement with the view of many workers that the bile plays a major rôle in the excretion of cholesterol.

It would perhaps have been desirable to have had more material on which to base the foregoing conclusions. Circumstances made it necessary, however, to interrupt the work at this point and it was thought best to publish the data thus far obtained, since the results were in good agreement throughout.

SUMMARY.

1. Dogs, from the intestines of which bile was excluded, have been maintained on strictly lipid-free diets for periods of time up to 5 weeks in length. Under these conditions they excrete lipids in amounts ranging from 1.5 to 4.5 times the average excretion while normal.

2. As in the normal dog there is considerable variation in the total excretion from week to week but no diminution in amount may be seen in the long continued series. The composition is quite constant, the average being 31.4 per cent unsaponifiable matter and 63.1 per cent fatty acids, containing 41.9 per cent solid and 45.5 per cent liquid acids.

3. Because of the close similarity in composition of the excretions of normal and bile fistula dogs the conclusion is drawn that the bile is not the source of fecal lipids. Various applications of the results to problems of cholesterol metabolism are discussed.

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XUM

THE DETERMINATION OF THE AMOUNT OF CONNECTIVE TISSUE IN MEAT.

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The desirability of any cut of meat as an article of diet depends largely upon the flavor and the toughness of its lean. While no appreciable headway has been recorded in the quantitative study of meat flavor and of the constituents of meat to which it is due, a study of the toughness of meat has been carried on for a number of years with notable success by Lehmann of the Hygienic Institute at Würzburg, Germany. In collaboration with a number of his pupils, Lehmann has shown (1) that the toughness of different cuts of meat, measured mechanically, was closely related to their content of connective tissue, and that the decrease in toughness resulting from cooking was related to the collagen of connective tissue rather than to the elastin. Under the influence of moist heat the collagen is readily changed to gelatin, thus losing its toughness. In the raw condition, white fibrous connective tissue (mainly collagen) is almost twice as tough as yellow elastic connective tissue (mainly elastin), but when cooked, the former loses most of its toughness while the latter remains practically unchanged in this respect. Physical differences in the muscle fibers themselves were not found to be appreciable factors in determining differences in toughness of muscle samples.

There is an obvious advantage in the use of a chemical measure of toughness as compared with a mechanical one, since Lehmann found that results of the mechanical test were so variable that a series of ten to twenty individual determinations should be run in order to obtain a representative average value. When only a limited amount of meat is available, therefore, it may be impossible to use the mechanical test to advantage.

Two methods were used in determining the connective tissue content of different muscles. One was a purely mechanical method developed by Schindler (2) in which a weighed portion of meat was simply scraped parallel with the fibers with a moderately sharp knife. The muscle cells are said to be easily scraped away, leaving a white framework of connective tissue fibers, which may be weighed either in the moist or in the dry condition.

In the same laboratory, Schepilewsky (3) worked out a chemical method for determining the connective tissue in flesh. In this method the muscle tissue is removed largely by mechanical means, consisting of trituration in a mortar with water, and sieving. The last traces are removed by treatment for 15 hours with cold 5 per cent sodium hydroxide solution. The separation of collagen and elastin is accomplished by treatment with hot alkali, in which the collagen is converted to gelatin and goes into solution.

We have followed the Schepilewsky method on a number of samples of meat and on white tendon obtained from pig shanks. We have found that a satisfactory filtration of the cold alkaline solutions is difficult, and we have obtained clear indications that collagen is appreciably soluble in 5 per cent sodium hydroxide, the solution used to remove the last traces of muscle tissue. Two samples of beef rib were submitted to the Schepilewsky method and to the method developed in this laboratory, which will be described later; in both cases, the gelatin results of the Schepilewsky method were only a small fraction of the results of the new method in which no alkaline extraction is used. A sample of white tendon was obtained by freezing pig tendons and slicing them in the frozen condition into thin sections. The sliced tendon (30 gm.) was then thoroughly macerated for 90 minutes with 300 cc. of water in a small ball mill. The water treatment and the sieving, followed by the treatment with cold alkali, was done according to Schepilewsky's directions. The filtrate, however, was cloudy, and contained over 50 per cent of the total nitrogen of the tendon. Upon acidification with acetic acid, a white cloudy precipitate separated, which was filtered off. The filtrate was clear and gave a deep color with the biuret test. Although no quantitative determination of the nitrogen contained in this clear solution was made, it was clearly evident that con-

siderable amounts of collagen nitrogen had been dissolved in the cold alkali.

It seems evident that a sharp separation of residual muscle stroma proteins from the characteristic proteins of connective tissue, collagen and elastin, is difficult if not impossible of attainment by either physical or chemical means. However, a more satisfactory separation than that accomplished by treatment with cold 5 per cent alkali would seem to be essential. We have adopted the following procedure. After the removal of most of the muscle tissue by cold water extraction and mechanical separation on a sieve, according to a method equivalent to that of Schepilewsky, the collagen is converted into gelatin by heating in the autoclave, and is removed by exhaustive hot water extraction. There is no reason to suspect that the water-insoluble proteins of the residual muscle tissue would be soluble in hot water. The residue remaining from this treatment is then digested with an alkaline trypsin solution at approximately 40°C. The muscle proteins are readily digested by trypsin while elastin is quite resistant.

In 1910, Baumstark and Cohnheim (4) showed that *in vitro* digestion of small pieces of meat (2 to 3 cm. long and 1 cm. in diameter) with pancreatic juice for 36 hours, removed the muscle fibers, leaving the connective tissue framework apparently intact. They concluded that digestion of connective tissue *in vivo* must be brought about by pepsin either in the stomach or in the upper part of the small intestine. The great resistance of elastin to tryptic digestion was amply confirmed by Abderhalden and Strauch (5) and Abderhalden and Meyer (6). In these investigations it was further shown that elastin readily adsorbs pepsin in the stomach and that the hydrolysis of elastin by this adsorbed pepsin continued throughout the length of the small intestine. Thus, elastin and possibly also collagen, are digested *in vivo* almost entirely through the action of the gastric protease, pepsin.

Trypsin does slowly attack elastin (5, 7), so that the separation of muscle proteins and elastin by means of tryptic digestion is only an approximation, the success of which will depend upon the choice of a time of reaction such that the error is least. The digestion of meat *in vitro* by trypsin in alkaline solution fully confirmed the results of preceding investigators. In 12 hours,

the muscle tissue was obviously largely if not entirely removed, leaving the white fibers of connective tissue. In the case of a sample of lean meat from the ribs of a 3 year old steer, it was found that at the end of 16 hours digestion, 36.3 per cent of the total nitrogen was undissolved, at the end of 24 hours, 32.8 per cent remained, and at the end of 40 hours, 27.5 per cent. A digestion period of 16 hours was adopted.

The filtration of the tryptic digest presented difficulties, since the alkali could not be neutralized without bringing down a precipitate of partially hydrolyzed protein. The expedient was finally adopted of filtering through a 120 mesh sieve.

While it might be considered that the connective tissue proteins might well be determined simply by means of the trypsin digestion, since collagen as well as elastin is extremely resistant to tryptic action (8), our attempts to devise such a method were not particularly successful. Satisfactory duplicates were difficult to obtain. Furthermore, since the separation is only approximate under the best of conditions, it would seem that the error would be the less the smaller the amounts of the separable proteins to be digested.

Description of Method.

The method finally adopted for the determination of the characteristic proteins of connective tissue in meat may be described as follows: Free the meat from all visible fat and surrounding connective tissue. Grind the sample through a meat chopper using a medium cutting plate. All meat remaining in the mill should be removed and thoroughly mixed with the ground sample.

Duplicate samples varying in weight from 25 to 100 gm. each may be used. The more connective tissue there is in the sample the smaller need the sample be. Place the sample in a small ball mill with 300 cc. of distilled water and macerate for 90 minutes. Transfer the sample onto a 40 mesh sieve, rejecting the filtrate. Wash the residue by taking up in 150 to 300 cc. of cold water, stirring thoroughly in a beaker, and filtering through the 40 mesh sieve. Repeated tests have shown that seven washings are sufficient to remove practically all of the water-soluble protein and most of the finely divided granular material.

Transfer the residue from the sieve to an 800 cc. beaker. Bring the volume up to about 400 cc., cover with a watch-glass, and heat for 2 hours in an autoclave under 16 to 18 pounds pressure. Release the pressure in the autoclave gradually and do not disturb the beakers for at least 5 minutes after the door is opened, for as long as the contents of the beakers are superheated there is danger of loss. Decant the hot supernatant liquid through a fluted filter paper, collecting the filtrate in a 1 liter volumetric flask. Wash the residue on the filter back into the beaker with 100 cc. of hot water, boil for a few minutes, and filter again. Repeat this process five times, or until the washings give only a constant faint color with the biuret test. Make the combined filtrates in the volumetric flask up to the mark and take aliquots for total nitrogen determinations.

Wash the residue on the filter back into a 400 cc. beaker with 100 cc. of cold trypsin solution.[1] Add 3 cc. of a mixture of chloroform and toluene and digest for 16 hours in the constant temperature oven at 38–40°C. Heat the solution to boiling, filter the trypsin extract through a 120 mesh sieve, and wash the residue three times with hot water, filtering each time through the sieve. Reject the filtrates. Determine total nitrogen in the residue by transferring to a Kjeldahl flask with water, digesting in the usual manner, and taking aliquots for distillation.

The nitrogen taken out by treatment in the autoclave and extraction with hot water is considered to be collagen nitrogen, and the nitrogen in the final residue, elastin nitrogen. The results are conveniently expressed in percentages of the total nitrogen of the meat.

There may be some question as to whether the results obtained for elastin may not include some of the other proteins of connective tissue. The coagulable proteins and the nucleoproteins of connective tissue, in so far as they are not removed by sieving in the initial process of the method, would presumably be dissolved during the tryptic digestion. The mucoid of connective tissue may also be dissolved at this stage, though no positive statement as to this can be made. In any case, the mucoid contamination

[1] The trypsin solution is made as follows: 1.5 gm. of powdered trypsin (we use a product put out by Fairchild Brothers and Foster of New York) and 6 gm. of sodium carbonate dissolved in 2 liters of water.

TABLE I.

Connective Tissue Proteins in Meat.

Sample No.	Description of sample.	Total nitrogen in sample.	Collagen nitrogen in per cent of total.	Elastin nitrogen in per cent of total.	Collagen + elastin nitrogen in per cent of total.
		per cent			
1	Beef rib.	3.19	8.4	6.4	14.8
			7.9	7.2	15.1
2	" "	3.29	2.5	8.1	10.6
			3.0	8.0	11.0
3	" "	3.65	9.4	4 7	14.1
			9.1	5.1	14.2
4	" "	3.35	4.2	8.7	12.9
			4.2	8.2	12.4
5	" shank.	3.42	7.5	14.4	21.9
			6.2	12.0	18.2
6	Pork tenderloin.	3.68	3.0	1.7	4.7
			2.3	1.8	4.2
7	Chicken, composite boneless meat from 2 lb. cockerels.	3.63	19.6	5.2	24.8
8	Chicken, composite boneless meat from 2 lb. pullets.	3.48	17.8	3.7	21.5
			18.0	4.1	22.1
9	Chicken, composite boneless meat from 3 lb. pullets.	3.28	15.1	1.0	16.1
			14.4	0.6	15.0
10	Chicken, composite boneless meat from 4 lb. cockerels.	3.61	20.5	0.6	21.1
			20.8	0.5	21.3
11	Chicken, breast muscle from 3 lb. cockerel.	3.24	2.1	0.8	2.9
			1.1	0.6	1.7
12	Chicken, thigh muscle from 3 lb. cockerel.	3.21	2.4	3.7	6.1
			2.0	6.5	8.5

TABLE I—*Concluded.*

Sample No.	Description of sample.	Total nitrogen in sample.	Collagen nitrogen in per cent of total.	Elastin nitrogen in per cent of total.	Collagen + elastin nitrogen in per cent of total.
		per cent			
13	Chicken, breast muscle from 3 lb. pullet.	4.06	1.7 3.4	 0.3	 3.7
14	Chicken, thigh muscle from 3 lb. pullet.	3.23	12.2 12.4	1.7 1.7	13.9 14.2
15	Chicken, breast muscle from 4 lb. cockerel.	4.14	6.5 6.8	1.6 1.6	8.1 8.4
16	Chicken, thigh muscle from 4 lb. cockerel.	3.69	11.9 13.5	1.8 2.4	13.8 15.9
17	Pork, white tendon.	5.50	87.7 90.7	4.8 4.1	92.5 94.8
18	Beef, " "	6.98	85.6 86.3	0.4 0.4	86.0 86.7
19	" yellow " ligamentum nuchæ.	6.97	17.9 17.4	80.0 80.0	97.9 97.4
20	Pork, water- and fat-free adipose tissue.	14.74	32.4 34.5	25.2 26.1	57.6 60.6

of elastin would probably not detract greatly from the relative significance of the results obtained.

Results Obtained by the Method.

A number of samples of animal tissue were analyzed according to the method described above. In the tabulation of these results, the duplicate determinations are given as illustrations of the agreement to be expected. All of our results to date with this method are included in Table I, though obviously a few of them in routine work would call for repetition.

The method is evidently not capable of detecting small differences in the connective tissue content of different samples of

meat. However, it would appear to be sufficiently accurate for many practical purposes. For example, a rib of beef is a tenderer cut than a shank, and the figures in the table indicate a distinctly smaller content of connective tissue, though different cuts of the same description apparently vary greatly in this respect, probably because of differences in condition of the steers from which they are taken. Pork tenderloin also evidently ranks as a very tender cut, according to the analysis of Sample 6. A comparison of Samples 7 to 10, inclusive, indicates that the composite sample of cockerel flesh is somewhat tougher than the composite sample of pullet flesh, while a comparison of Samples 11 to 16 inclusive, shows that the thigh of a chicken carries distinctly tougher meat than the breast. The latter samples do not indicate any clear cut differences between cockerel and pullet flesh.

The results for Samples 17, 18, and 19 permit a rough comparison with the results of other methods of separation. Buerger and Gies (9) report the composition of the organic matter of white tendinous tissue from the ox as follows: 85.1 per cent of collagen, 4.4 per cent of elastin, 3.5 per cent of mucoid, 0.59 per cent of coagulable proteins, 2.8 per cent of ether-soluble matter, and 2.4 per cent of extractives and undetermined constituents (obtained by difference). Vandegrift and Gies (10) give the following as the average composition of the organic matter of the ligamentum nuchæ of the ox: 74.6 per cent of elastin, 17.0 per cent of collagen, 1.2 per cent of mucoid, 1.5 per cent of coagulable protein, 2.6 per cent of ether-soluble matter, and 1.9 per cent of undetermined material.

The results obtained on Sample 20 may not be representative of ordinary connective tissue, since the sample was analyzed in a dry, finely ground condition, permitting the possibility of appreciable loss of collagen or elastin elements through the 40 mesh sieve.

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THE DISTRIBUTION OF DIHYDROSITOSTEROL IN PLANT FATS.*

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INTRODUCTION.

It has been shown in previous reports from this laboratory that the fat extracted from the endosperm and bran of corn (1) and wheat (2) contained a saturated sterol, dihydrositosterol, $C_{27}H_{47}OH$, in addition to unsaturated sterols. To what extent dihydrositosterol is distributed in plant fats is not known but it seemed probable that it would be found in the fat obtained from the endosperm and bran of various seeds. The present paper gives additional information on the distribution of dihydrositosterol.

The unsaponifiable matter obtained from the fat extracted from cottonseed meal and linseed meal has been examined for dihydrositosterol. If the saturated sterol is concentrated in the outer coverings of these seeds, as is the case with corn and wheat, it was thought that the fat remaining in the meal after the oils had been pressed out might be richer in dihydrositosterol than the oils obtained by expression. This was not found to be the case to any marked degree at least, for the sterols isolated from the fat extracted from the meals were very similar to those obtained from the pressed oils.

The fat extracted from rice bran is rich in unsaponifiable matter but the yield of crystalline sterols is very small because the greater portion of the material consists of a brown viscous liquid. Wein-

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hagen (3) examined the rice bran fat and described the crystalline sterols as sitosterol. The sterol fraction has been more fully described in another paper (4). It will suffice to state here that at least three different sterols were present and from this mixture a small quantity of dihydrositosterol was isolated.

The unsaponifiable matter obtained from corn oil has been examined by several investigators (5). Since the paper published by Gill and Tufts (6) the sterol has generally been regarded as identical with the sitosterol prepared from wheat and rye germ by Burian (7).

For the present investigation about 100 gm. of crystalline sterols were prepared from 10 kilos of corn oil. It was believed that the substance was pure sitosterol but investigation showed that the material could be separated by crystallization into a number of fractions that varied greatly in optical rotation (8). From the top fraction 1.65 gm. of pure dihydrositosterol were obtained by the method of Anderson and Nabenhauer (9).

Since dihydrositosterol was found in the oil prepared from corn germ it was decided to examine wheat germ oil (10) for the same substance. The sterols of wheat germ and rye germ were first investigated by Burian (7) and somewhat later by Ritter (11). The substance was called sitosterol and except for a very small amount of material obtained from the mother liquors called parasitosterol, it was supposed to represent a homogeneous compound. For the present work commercial wheat germ was secured which appeared to be very pure but a microscopic examination revealed a slight admixture of fine particles of bran and endosperm. Since earlier investigations have shown that wheat endosperm and wheat bran contain relatively large quantities of dihydrositosterol, one might with reasonable certainty predict the presence of a small amount of this sterol in the unsaponifiable matter derived from the wheat germ described above. The fat extracted from 44 kilos of the wheat germ gave on saponification 158 gm. of unsaponifiable matter from which 0.44 gm. of dihydrositosterol was obtained. Through the cooperation of Evans and Burr 100 gm. of crude crystalline wheat germ sterols, obtained as by product in their preparation of vitamin E (12) were also examined. From this material 1.65 gm. of pure dihyrositosterol were isolated.

The results that have been obtained so far indicate that di-

hydrositosterol is widely distributed in plant fats. It has been isolated from the fat from rice bran and it occurs not only in the endosperm and bran of corn and wheat but small amounts have been obtained from the oils prepared from the germ of these grains. To what extent dihydrositosterol would be present in absolutely pure wheat germ is unknown but it would be very difficult to secure a sufficient amount of such pure material.

There is no easy or convenient method of isolating dihydrositosterol. It has been found necessary to recrystallize the crude sterols until the top fraction shows only a slight levorotation. The substance is then dissolved in carbon tetrachloride and treated with acetic anhydride and sulfuric acid as described by Anderson and Nabenhauer (9). With certain sterol mixtures this method works very well but occasionally it is quite unsatisfactory, since practically all of the sterols may disappear from the reaction mixture evidently due to the formation of sulfonic acids or sterol sulfates. These compounds are soluble in water and hence are removed from the carbon tetrachloride when the latter is washed with water. They also form very stable emulsions when the solvent is shaken with water which renders the separation of the two liquids very difficult or impossible.

To obviate some of these difficulties it has been found necessary to concentrate the saturated sterol in the top fraction by crystallization from ethyl alcohol until the rotation is close to 0°. The resulting mixture which is rich in the saturated sterol is then acetylated, dissolved in carbon tetrachloride, and treated with acetic anhydride and sulfuric acid. The formation of saturated sterol sulfates appears to be greatly reduced by this procedure; a better separation and an increased yield of dihydrositosterol is therefore secured.

The functions of dihydrositosterol in plant metabolism are unknown but it is interesting to note that it is the analogue of coprosterol (13), the saturated sterol which is presumably a reduction product of cholesterol, which is eliminated in the feces of human beings and of certain animals. The experiments of Hess and Weinstock (14) have shown that dihydrositosterol differs in physiological activity from unsaturated sterols in that the substance acquires no antirachitic property on irradiation with ultraviolet light.

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The dihydrositosterols that have been isolated from different sources are all very similar in their properties but it cannot be stated definitely that they are identical chemical compounds. Some differences in the melting point of the free sterols as well as of the acetyl derivatives have been noted, yet no depression of the melting points of mixtures of these substances was obtained. Slight differences in the magnitude of the optical rotations have also been observed. The variations may be due to differences in

TABLE I.

Properties and Composition of Dihydrositosterol.

Source.	Composition.		M.p.*	$[\alpha]_D$†	Acetate, m.p.	$[\alpha]_D$
	C	H				
	per cent	*per cent*	*°C.*		*°C.*	
Corn.						
Gluten	83.42	12.46	143–144	+24.23°	137	+14.41°
Bran	83.30	12.23	142–143	+24.05°	139	
Oil	83.13	12.54	143–144	+25.18°		
"	83.49	12.66	143–144	+23.81°		
Wheat.						
Bran	83.34	12.64	145–146	+25.82°	140	
"	83.34	12.22	144–145	+23.79°	140	+12.78°
Germ	83.07	12.75	143–144	+22.20°		
"	83.26	12.61	143–144	+23.61°	141	+12.72°
Rice bran	83.02	12.24	144–145	+23.90°		
Sitostanol	83.39	12.44	142–143	+24.16°	139	
Calculated for $C_{27}H_{47}OH$	83.50	12.37				

* Melting points mentioned in this paper are corrected.

† All determinations of optical rotations were made in chloroform solution and with sodium light.

purity but in view of the large number of isomers that are possible among the sterols one can easily imagine the possibility of the existence of many isomeric compounds of this class that would probably differ but slightly in physical properties.

The dihydrositosterol that occurs in plant fats appears to be identical with the synthetic sitostanol that is obtained when an unsaturated sterol, possessing the properties usually ascribed to sitosterol, is reduced with hydrogen in the presence of platinum black. At least the melting point and optical rotation of these compounds are very similar.

The properties and composition of the various dihydrositosterol preparations that have been isolated are summarized in Table I in comparison with the synthetic sitostanol.

EXPERIMENTAL PART.

Examination of the Sterols from Cottonseed Meal.

Cottonseed meal, 30 kilos, was extracted with petroleum ether. The oil that remained on evaporating the solvent, 886 gm., was saponified with alcoholic potassium hydroxide. The soap solution was diluted with water and extracted with 4 portions of ether. After the ethereal solution had been washed with water the ether was distilled, and the residue was dried. The crude unsaponifiable matter weighed 13 gm. which is equal to 1.46 per cent of the oil. The dark colored semisolid mass was stirred up with a little cold petroleum ether, filtered, and washed with cold petroleum ether. The nearly colorless crystals thus obtained weighed 4.45 gm. The substance was recrystallized from 200 cc. of alcohol when 3.3 gm. of colorless crystals were obtained. The mother liquor gave 0.65 gm. of colorless crystals on concentration. The rotation of the first fraction was $-34.05°$ and the second fraction was $-35.24°$. These values are similar to those found with the sterols prepared from cottonseed oil (15) and would indicate that cottonseed meal does not contain any greater proportion of dihydrositosterol than does the oil.

Sterols from Linseed Meal.

By the method outlined above 15 kilos of linseed meal gave 927 gm. of crude fat which yielded after saponification and extraction with ether 17 gm. of unsaponifiable matter in the form of an oily mass. A small amount of crystalline sterols was obtained by dissolving the oil in alcohol and allowing the solution to cool. The crude crystals were filtered, washed with cold petroleum ether, and recrystallized from alcohol after treatment with norit. About 1 gm. of colorless crystals was obtained, m.p., 137–138°; $[\alpha]_D$, $-33.16°$. These values are similar to those observed with the sterols prepared from linseed oil, and evidently linseed meal does not contain any appreciable quantity of dihydrositosterol.

Dihydrositosterol from Rice Bran Fat.

Rice bran, 18 kilos, was extracted in the manner described above, giving 1800 gm. of crude fat which after saponification and extraction with ether yielded 95 gm. of unsaponifiable matter. The substance was a brown viscous oil from which 9 gm. of crystalline sterols were obtained with difficulty, using acetone and alcohol as solvents. This sterol had a rotation of $-27.67°$ and after 5 further recrystallizations the top fraction weighed 3 gm., $[\alpha]_D$, $-22.94°$. The substance was dissolved in carbon tetrachloride and the solution was treated with acetic anhydride and sulfuric acid several times until the unsaturated sterols were removed. The solvent was distilled and the residue was boiled with alcoholic potassium hydroxide. The solution was diluted with water, extracted with ether, and the ether was distilled. The residue was treated with norit and recrystallized several times from alcohol. The substance separated in colorless hexagonal plates, m.p., 144–145°; $[\alpha]_D$, $+23.90°$.

The substance was dried for analysis at 105° in a vacuum over phosphorus pentoxide. The loss in weight on drying was 4.57 per cent, which corresponds to 1 molecule of water of crystallization.

0.0564 gm. substance: 0.0617 gm. H_2O and 0.1717 gm. CO_2.
Calculated for $C_{27}H_{47}OH$ (388). C 83.50, H 12.37 per cent.
Found. C 83.03, H 12.24 per cent.

The properties and composition of the substance indicate that it was dihydrositosterol. The quantity of pure substance obtained was only 0.1 gm. or about 1 per cent of the crystalline sterols but a larger proportion must have been present in the unsaponifiable matter because considerable losses are bound to occur in the isolation and purification.

Dihydrositosterol from Corn Oil.

Refined corn oil,[1] 10 kilos, was saponified and the unsaponifiable matter was isolated as described above and recrystallized from alcohol. After eighteen recrystallizations the top fraction weighed 19.7 gm., m.p., 139–140°; $[\alpha]_D$, $-17.45°$. Dihydrositosterol was

[1] Supplied by the Patent Cereals Company, Geneva, N. Y.

isolated by the method described above. Owing to the large percentage of unsaturated sterols that were present repeated treatments with acetic anhydride and sulfuric acid were necessary and in the subsequent washing with water very stable emulsions were formed which undoubtedly occasioned serious losses of the saturated sterol. From 5 gm. of the top fraction only 0.25 gm. of pure dihydrositosterol was obtained. The balance of the top fraction was recrystallized many times and finally acetylated. The acetyl derivative was dissolved in carbon tetrachloride and the solution was treated with acetic anhydride and sulfuric acid when 1.4 gm. of pure dihydrositosterol were obtained. Calculated from the rotation (16), which was −17.45°, the top fraction should have contained about 6 gm. of dihydrositosterol.

The pure dihydrositosterol obtained as mentioned above crystallized in large colorless hexagonal plates. It melted at 143–144°, solidified at 140°, and remelted at 144–145°; $[\alpha]_D$, +23.81°.

The substance was dried for analysis at 105° in a vacuum over phosphorus pentoxide. The loss in weight was 4.54 and 4.60 per cent which corresponds to 1 molecule of water of crystallization.

0.1094 gm. substance: 0.1238 gm. H_2O and 0.3349 gm. CO_2.
Calculated for $C_{27}H_{47}OH$ (388). C 83.50, H 12.37 per cent.
Found. C 83.49, H 12.66 per cent.

Dihydrositosterol from Wheat Germ Oil.

The wheat germ[2] used in this work appeared to be as pure as this material can be obtained commercially. Microscopic examination revealed some particles of bran and also a small amount of starch. From 44 kilos of germ nearly 4 kilos of oil were obtained by extraction with ether containing 10 per cent of alcohol. The oil was saponified and the unsaponifiable matter was isolated as described above. The substance was recrystallized thirty-six times from alcohol and the top fraction then weighed 3.1 gm. It melted between 130–133°; $[\alpha]_D$, −3.18°. This material was acetylated and the acetyl derivative was dissolved in carbon tetrachloride and treated with acetic anhydride and sulfuric acid. It was necessary to repeat this treatment eight times, using 10 cc. each of the reagents, before the Liebermann-Burchard reaction

[2] Supplied by the Pillsbury Flour Mills Company, Buffalo, N. Y.

was negative. The solvent was then washed with water, evaporated, and the residue was saponified with alcoholic potassium hydroxide. The sterol was precipitated by adding water, filtered, washed with water, and dried. It was dissolved in alcohol, treated with norit, and recrystallized four times. The colorless plates that were obtained weighed 0.44 gm., m.p., 143–144°; $[\alpha]_D$, +22.20°.

When the substance was dried at 105° in a vacuum over phosphorus pentoxide the loss in weight was 3.25 per cent which is somewhat lower than the calculated value for 1 molecule of water of crystallization.

0.1128 gm. substance: 0.1286 gm. H_2O and 0.3436 gm. CO_2.
Calculated for $C_{27}H_{47}OH$ (388). C 83.50, H 12.37 per cent.
Found. C 83.07, H 12.75 per cent.

Another lot of unsaponifiable matter prepared from wheat germ fat was also examined for dihydrositosterol. The material consisted of 100 gm. of crude crystalline sterols obtained as a by product by Evans and Burr in their preparation of vitamin E. The substance which was slightly yellow was boiled with alcoholic potassium hydroxide for 2 hours. The sterols were isolated in the usual manner by extraction with ether and recrystallization from alcohol. The top fraction weighed 23.3 gm. after the substance had been recrystallized fifteen times. It melted at 138–139°; $[\alpha]_D$, −18.45°. Attempts to isolate dihydrositosterol after this product had been treated in carbon tetrachloride solution with acetic anhydride and sulfuric acid were not very successful. Repeated treatments with the reagents were necessary before a colorless solution was obtained and as a result nearly all of the dissolved sterols disappeared. The saturated sterol evidently formed a sulfate in the above reaction which either separated in the acid layers together with the unsaturated sterol compounds or else it was lost in washing the carbon tetrachloride solution with water.

The balance of the top fraction, which now weighed 15.3 gm., was again recrystallized ten times in order to remove the unsaturated sterol as much as possible. The substance weighed 3.6 gm., m.p., 138°; and it was optically inactive. It was acetylated and the acetyl derivative was treated in carbon tetrachloride solution with acetic anhydride and sulfuric acid as described above.

After the solvent had been washed with water it was distilled and the residue was saponified with alcoholic potassium hydroxide. The sterol was isolated and recrystallized as described before. It weighed 1.6 gm. and crystallized in colorless hexagonal plates. In the Liebermann-Burchard reaction it gave no immediate coloration but after standing for some time a faint green color developed. The substance melted at 143–144°, solidified at 138–139°, and remelted at 144°; $[\alpha]_D$, +23.61°.

For analysis the substance was dried at 105° in a vacuum over phosphorus pentoxide. It lost 4.79 per cent which corresponds to 1 molecule of water of crystallization.

0.1095 gm. substance: 0.1234 gm. H_2O and 0.3343 gm. CO_2.
Calculated for $C_{27}H_{47}OH$ (388). C 83.50, H 12.37 per cent.
Found. C 83.26, H 12.61 per cent.

Preparation of the Acetyl Derivative.

The acetyl derivative was prepared by boiling 0.6 gm. of the sterol with 10 cc. of acetic anhydride for 1 hour. After the solution had cooled the crystals were filtered and washed with methyl alcohol. It was recrystallized from ethyl alcohol from which it separated in large colorless plates. The substance melted at 141°, solidified at 134°, and remelted at 141°; $[\alpha]_D$, +12.72°. There was no loss in weight on drying at 105°.

0.1108 gm. substance: 0.1181 gm. H_2O and 0.3283 gm. CO_2.
Calculated for $C_{27}H_{47}O \cdot CO \cdot CH_3$ (430). C 80.93, H 11.62 per cent.
Found. C 80.81, H 11.92 per cent.

It is evident from the data that have been presented that the crystalline sterols that were examined contained not less than 1.6 per cent of dihydrositosterol. The amount of saturated sterol actually present in the above preparation must have been greater than that indicated because considerable losses occur during the isolation.

In conclusion the authors desire to express their appreciation to Dr. G. O. Burr of the University of California, for a generous supply of crude wheat germ sterols.

SUMMARY.

1. The saturated sterol, dihydrositosterol, $C_{27}H_{47}OH$, is apparently rather widely distributed in plant fats.

2. It occurs in association with unsaturated sterols, not only in the endosperm and bran of corn and wheat, but also in the oils obtained from the germ of these grains.

3. Appreciable quantities of the substance have been isolated from corn gluten, corn bran, wheat bran, rice bran, corn oil, and wheat germ oil.

4. The dihydrositosterol preparations that have been obtained from different sources show slight variations in physical properties. The melting points vary from 141–142° to 145–146° and the specific optical rotations vary from about +23° to +25°. The acetyl derivatives vary in melting points from 137–141° and in optical rotation from about +13° to +14°. Whether these variations in properties depend upon the degree of purity or if they are due to the presence of isomeric saturated sterols, cannot be determined from the present data

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THE REDUCTION PRODUCTS OF CERTAIN PLANT STEROLS.

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INTRODUCTION.

It was first shown by Willstätter and Meyer (1) that the normal saturated sterol, β-cholestanol, was formed when cholesterol in ethereal solution was reduced with hydrogen in the presence of platinum black. Since that time a number of experiments has been reported in which plant sterols have been reduced with hydrogen in the presence of a catalyst but only a few reduction products of apparently normal phytosterols having one double bond have been described. The first experiments in this direction were reported by Menozzi and Moreschi (2) who reduced a phytosterol preparation isolated from walnut oil. Windaus and Rahlen (3) reduced sitosterol obtained from Calabar beans with hydrogen and colloidal palladium. Anderson and Nabenhauer (4) followed the method of Willstätter and Meyer (1) but used the catalyst described by Voorhees and Adams (5) in reducing a phytosterol prepared from wheat bran. All of the reduced plant sterols prepared by the investigators mentioned above varied in properties as is shown in Table I.

The variations in properties might have been due to incomplete reduction but this cause must be ruled out since it was stated in every case that the usual sterol color reactions were negative. Another important factor influencing the properties of the reduction product would be the purity or homogeneity of the sterols that were used in these experiments.

In the search for a pure and homogeneous sitosterol which has been carried on in this laboratory during the past 2 years, a con-

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siderable number of sterol fractions has been reduced. These reduction products have been briefly referred to in other publications (6) but since the results might be of interest to other workers a fuller description is presented in this paper.

It has been indicated in a previous publication (7) that the natural dihydrositosterol isolated from various plant fats varied slightly in physical properties and that it was uncertain whether the observed variations were due to differences in purity or to the occurrence of isomeric compounds. In the work on the sterols isolated from corn oil (8) and wheat germ oil (9) it was shown that the sitosterol fraction consisted of at least three isomeric sterols which were designated α-, β-, and γ-sitosterol. The only one of these isomers for which any claim to purity could be made was γ-sitosterol.

TABLE I.

Properties of Dihydrophytosterols.

Authors.	M.p.	$[\alpha]_D$ in $CHCl_3$.	Acetate, m.p.
	°C.		°C.
Menozzi and Moreschi	136–137	+22.97°	134–135
Windaus " Rahlen	137	+27.90°	132
Anderson and Nabenhauer	142–143	+24.16°	138–139

The α and β fractions were obviously mixtures that could not be separated by any method available at present. The substance designated α-sitosterol contained a relatively large amount of β- but only a small quantity of γ-sitosterol. The β fraction was free from α- but contained a small amount of γ-sitosterol. It is interesting therefore that the α- and β-sitosterol fractions, which were not homogeneous, gave reduction products that were apparently identical and that were very similar to the natural dihydrositosterol. The dihydro-γ-sitosterol, however, differed decidedly from these preparations in optical rotation.

Unless other investigators have been more fortunate in securing homogeneous preparations of sitosterol, it is likely that many of the compounds that have been described have been mixtures. Perhaps the only reasonably pure reduced plant sterols obtained so far are stigmastanol (10) and γ-sitastanol.

EXPERIMENTAL PART.

The platinum catalyst used in all of these experiments had been prepared according to the method of Voorhees and Adams (5). The apparatus was similar to that already described (4).

n-Butyl alcohol is a good solvent for sterols but it is not suitable in reduction experiments because certain impurities, that apparently cannot be removed, destroy the catalyst. The unsaturated sterol was dissolved in ether or alcohol or in mixtures of these solvents but occasionally glacial acetic acid was used. The solution was mixed with 0.2 or 0.3 gm. of the catalyst; the apparatus was evacuated and hydrogen was introduced to a pressure of 15 to 20 mm. of mercury. The mixture was shaken while more hydrogen was added from time to time, maintaining the above mentioned pressure.

TABLE II.

Properties of Unsaturated Sterols before Reduction.

Name and source of sterol.	M.p.	$[\alpha]_D$
	°C.	
α-Sitosterol, corn oil	135–136	−13.45°
" wheat germ oil	138–141	−23.41°
β-Sitosterol, corn oil	139–140	−36.11°
" wheat germ oil	140	−35.06°
γ-Sitosterol, corn oil	145–146	−42.43°
" wheat germ oil	146–147	−41.32°
" " " "	147–148	−42.47°

The reduction was continued in every case until a sample, obtained by evaporating a portion of the solution, failed to give any immediate coloration in the Liebermann-Burchard reaction. The mixture was then shaken with air until the catalyst separated. The solution was filtered, concentrated by distillation, and the reduction product was recrystallized from alcohol.

The reduced sterols were all very similar in appearance and they were much less soluble in the usual solvents then the unsaturated compounds. They crystallized from alcohol in colorless plates that contained 1 molecule of water of crystallization. In the Liebermann-Burchard reaction no immediate coloration was obtained but when the reaction mixture was allowed to stand at

TABLE III.

Properties and Composition of Reduced Plant Sterols.

Name and source of sterol.	Composition.		M. p.	$[\alpha]_D$	Acetyl derivative.			
					Composition.		M. p.	$[\alpha]_D$
	C	H			C	H		
	per cent	*per cent*	*°C.*		*per cent*	*per cent*	*°C.*	
α-Sitostanol.								
From corn oil	83.41	12.62	139–140	+23.53°	80.67	11.88	137–138	+13.64°
" wheat germ	83.24	12.66	137–138	+23.20°	80.68	11.62	136–137	+13.69°
β-Sitostanol.								
From corn oil	83.46	12.65	140–141	+24.91°	80.68	12.06	136–137	+14.32°
" wheat germ	83.60	12.74	139–140	+24.22°	81.20	11.85	137–138	+13.57
γ-Sitostanol.								
From corn oil	83.61	12.58	144–145	+17.82°	80.65	11.88	143–144	+8.98°
" wheat germ	83.37	12.56	143–144	+18.01°	80.64	11.84	142–143	+9.98°
" " "	83.59	12.64	144–145	+17.88°			144–145	+10.11°
" " "			143–144	+18.89°				
Calculated for $C_{27}H_{47}OH$	83.50	12.37						
Calculated for $C_{27}H_{47}O\cdot CO\cdot CH_3$					80.93	11.62		

room temperature a faint green color developed in the course of 5 or 10 minutes.

β- and γ-sitosterol were easily reduced as a rule but α-sitosterol was reduced much more slowly, repeated treatments with fresh portions of catalyst being necessary before the Liebermann-Burchard reaction became negative.

The properties of the unsaturated sterols that were used in the reduction experiments are given in Table II.

The preparations described in Table II were reduced and the reduction products were isolated and recrystallized as described above. The reduced sterols when dried at 105° in a vacuum over phosphorus pentoxide lost weight corresponding to 1 molecule of water of crystallization. The results are summarized in Table III.

An inspection of the data in Table III shows that α- and β-sitosterol, *i.e.* the bottom and intermediate fractions obtained on fractionation of the acetyl derivative, although varying greatly in properties yield practically identical reduction products. The sterols melt at about 140° and the rotation is about +24°, while the acetyl derivatives melt at about 137–138° and the rotation is about +14°.

Although γ-sitostanol melts several degrees higher than α- and β-sitostanol yet there is no depression of the melting point when mixtures of these substances are heated. The principal difference between these compounds is the lower dextrorotation of γ-sitostanol.

SUMMARY.

1. Three isomeric sitosterol preparations have been reduced with hydrogen in the presence of platinum black.

2. α- and β-sitosterols yield apparently identical α- and β-sitostanols, $C_{27}H_{47}OH + H_2O$, m.p., about 140°; $[\alpha]_D$, about +24°. The acetyl derivatives melt at about 137–138°; $[\alpha]_D$, about +14°.

3. The reduction product of γ-sitosterol is different from α- and β-sitostanols. γ-Sitostanol, $C_{27}H_{47}OH + H_2O$, melts at about 144–145°; $[\alpha]_D$, about +18°. The acetyl derivative melts at about 143–144°; $[\alpha]_D$, about +9° to +10°.

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PROPERTIES OF CHOLESTEROL OBTAINED FROM DIFFERENT SOURCES.

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INTRODUCTION.

The question of the origin of cholesterol has not been settled. Unicellular organisms are apparently capable of synthesizing sterols as well as other cell constituents from simpler organic compounds. In the case of animals it is believed that they utilize plant sterols (1) and that in some manner somewhere in the body phytosterol is changed to the isomeric compound, cholesterol, which only varies from the former in physical properties. Experimental evidence has also been presented (2) which indicates that cholesterol may be synthesized in the animal body.

It would seem, however, from recent work on plant sterols (3) that more far reaching molecular rearrangements must be necessary in order that phytosterols may be changed into cholesterol than has been supposed heretofore. To consider only one plant fat, for instance that from corn germ (4), it has been found that the crystalline sterol isolated from it is a complex mixture consisting of the saturated dihydrositosterol, the unsaturated stigmasterol with two double bonds, and at least three isomeric sitosterols with one double bond each. That these different phytosterols, which vary not only in physical properties but also in composition, could all be changed into one single cholesterol does not seem likely. It may be necessary therefore to reckon with the possibility of a number of isomeric cholesterols in animal fats.

In connection with the investigation conducted by Bloor (5) on the fatty acids of the plasma of beef and dog blood, considerable

quantities of unsaponifiable material were obtained. These constituents were kindly given us by Dr. Bloor for examination.

A decided difference in the physical appearance of the crude material obtained from beef and dog plasma was noticed, and even the purified cholesterols showed slight differences in melting points and in optical rotation. The substance from beef plasma consisted almost entirely of crystalline cholesterol which after it had been purified appeared to be homogeneous. Although the animals had received only plant sterols in their food, there was no evidence of the presence of phytosterols in the blood plasma. The transformation of phytosterols into cholesterol, if it occurs, must therefore take place very quickly.

The unsaponifiable matter from the plasma of dog blood contained some 40 per cent of non-crystalline semisolid material that could not be eliminated from the cholesterol by crystallization from alcohol. Precipitation with digitonin did not lead to a pure cholesterol because a part of the non-crystalline substance was also precipitated. Pure cholesterol could only be obtained by means of the dibromo- compound.

The fact that a cholesterol preparation appears to be homogeneous, *i.e.* recrystallization causes no change in the properties, is no absolute proof of the purity. Closely allied sterols may differ so slightly in solubility that recrystallization causes no perceptible separation. It has been shown that a mixture of isomeric sitosterols (6) may be recrystallized at least ten times without showing any notable change in properties.

In order to determine whether different cholesterol preparations are homogeneous it will be necessary to prepare cholesterol esters of various acids or some other derivatives of cholesterol and fractionally recrystallize these compounds, and to compare the properties of the different fractions. As a beginning in this direction we have subjected cholesterol acetate to fractional crystallization from ethyl alcohol. The results indicate that commercial cholesterol contains a small amount of some substance which causes a decided lowering of the melting point and of the optical rotation of the bottom fraction. More experimental work will be necessary before one can determine whether the depression of the melting point and optical rotation is due to the presence of an accidental impurity or if it is due to some compound isomeric with cholesterol.

EXPERIMENTAL PART.

Cholesterol from Beef Plasma.

The crude unsaponifiable matter was supplied by Dr. W. R. Bloor and was obtained as a by product from his work on the Fatty Acids of Blood Plasma (5). It had been prepared as follows: The plasma was digested in about 20 per cent sodium hydroxide at 90°C. for 8 hours, after which the mixture was cooled, acidified with hydrochloric acid, and the lipid material was extracted with ether. The fatty acids were separated from the unsaponifiable matter by first dissolving the mixture in petroleum ether and then adding successively equal volumes of alcoholic potassium hydroxide and of water. The fatty acids dissolved in

TABLE I.

Properties of Different Fractions of Cholesterol from Beef Plasma.

Fraction No.	Weight.	M. p.*	Solidifying point.	$[\alpha]_D$†
	gm.	*°C.*	*°C.*	
1	15.5	149	118	−39.52°
2	6.2	149	118	−39.40°
3	1.1	149	116	−39.71°

* All melting points given in this paper are corrected.

† All optical rotations were determined in chloroform solution and with sodium light.

the alkaline alcohol and water while the unsaponifiable substance remained in the petroleum ether. After a second extraction with petroleum ether the combined extracts were washed with water, the solvent distilled off, and the residue was dried.

The crude cholesterol, which weighed 25 gm., was yellow in color and consisted of large irregular plate-shaped crystals. The material was dissolved in 700 cc. of hot alcohol, boiled with norit, and filtered. As the solution cooled the cholesterol separated in very large, thin, colorless plates that filtered very slowly on a Buchner funnel. The substance was recrystallized three times, using about 600 cc. of alcohol for each crystallization. When the solution was cooled rapidly the cholesterol crystallized in small uniform plates. After drying at 60°C. the crystals weighed 15.5

gm. This is designated Fraction 1 in Table I. Fraction 2 was obtained by concentrating the above mentioned mother liquor and recrystallizing the substance four times. Fraction 3 was obtained on concentrating the last mother liquors and recrystallizing the product nine times until a colorless preparation resulted. The properties of these fractions are shown in Table I.

Fraction 1 was analyzed after it had been dried at 105°C. in a vacuum over phosphorus pentoxide.

Analysis.

0.1505 gm. substance: 0.1604 gm. H_2O and 0.4622 gm. CO_2.
Calculated for $C_{27}H_{45}OH$ (386). C 83.93, H 11.91 per cent.
Found. C 83.75, H 11.92 per cent.

The properties of the three fractions described in Table I were practically identical and the composition agreed with the usual formula for cholesterol.

TABLE II.

Optical Rotation of Cholesterol and Cholesteryl Acetate in Chloroform and Ether.

Cholesterol.			Cholesteryl acetate.		
$[\alpha]_D$ in $CHCl_3$.	$[\alpha]_D$ in ether.		$[\alpha]_D$ in $CHCl_3$.	$[\alpha]_D$ in ether.	
−39.52°	−31.46°	−31.60°	−43.11°	−30.90°	−30.54°

Acetyl Derivative.

The acetyl derivative was prepared by boiling 4 gm. of Fraction 1 with 30 cc. of acetic anhydride for 1.5 hours. After cooling, the crystals were filtered off, washed with cold methyl alcohol, and recrystallized three times from ethyl alcohol. The substance separated in fine colorless needles which after drying in the air weighed 3.7 gm. It melted at 116° and on cooling a fine display of colors was observed. The rotation was −43.11°.

Variation in Optical Rotation in Different Solvents.

The optical rotation of cholesterol and of cholesteryl acetate varies greatly with the solvent. A comparison of the optical activity in chloroform and ether is given in Table II.

The levorotation is depressed in ethereal solution and the introduction of the negative acetyl group which causes an increase in levorotation in chloroform gives a lower value in ether than does free cholesterol.

Reduction of Cholesterol.

β-Cholestanol has been prepared and studied by several investigators, having first been described by Diels and Abderhalden (7). It was first shown by Willstätter and Meyer (8) that the same substance was formed when cholesterol was reduced by hydrogen in the presence of platinum black. β-Cholestanol has since that time been prepared by a number of investigators (9) and the melting point is usually given as 141–142° while the recorded rotations vary from +23° to +34°.

The cholesterol, 5 gm. of Fraction 1, was reduced with hydrogen and platinum oxide as described for sitosterol (10). The β-cholestanol was recrystallized several times from alcohol from which it separated in large colorless plates containing 1 molecule of water of crystallization. In the Liebermann-Burchard reaction no immediate coloration was given but after the reaction mixture had stood for some time a faint bluish color developed. The substance melted at 143°. The rotation was determined in chloroform and in ether with the following results:

In chloroform solution, $[\alpha]_D$, +23.56°, +23.45°.
In ethereal solution, $[\alpha]_D$, +27.07°, +27.06°.

Acetyl Derivative of β-Cholestanol.

The acetyl derivative was prepared and twice recrystallized from alcohol. It melted at 111° and the rotation in chloroform solution was +13.27°.

Examination of Unsaponifiable Matter from Normal Dog Blood Plasma.

The crude material was supplied by Dr. W. R. Bloor and it had been obtained in a similar way to that from beef blood plasma. The substance, which weighed 16.3 gm., was a yellow crystalline solid that obviously contained much oily material. It possessed a

slight but peculiarly unpleasant odor. The substance was dissolved in 400 cc. of hot alcohol and treated twice with norit, which did not remove much of the coloring matter. After six recrystallizations the top fraction was snow-white, but the crystals were not homogeneous; two forms of crystals were evident, *viz.* colorless plates that somewhat resembled cholesterol and also branching fern-like crystals. The substance weighed 1.9 gm., m.p., 143–144°.

The yellow colored mother liquors from the above mentioned crystallizations now contained most of the original material in solution. It was again treated with norit, filtered, and concentrated to about 600 cc. On standing at room temperature 4 gm. of colorless plate-shaped crystals separated, m.p., 151–152°. The filtrate was concentrated to about 200 cc. and allowed to cool when 6 gm. of slightly yellow crystals were collected, m.p., 149–150°.

The last mother liquor deposited, on standing for several days, about 50 mg. of yellow octahedral crystals that were identified as sulfur. After the above mentioned crystals had been filtered off, the filtrate was evaporated to dryness when a yellow wax-like material of unpleasant odor remained.

The appearance of the various crystalline fractions, obtained as described above, together with the variations in melting point indicated that the material was very impure and it seemed to be impossible to obtain any pure cholesterol by crystallization. An attempt was therefore made to precipitate the cholesterol from the mixture by means of digitonin according to the method of Windaus (11). Portions of 4.5 gm. of the impure cholesterol were dissolved in 400 cc. of hot alcohol and mixed with a solution containing 15 gm. of digitonin in 1500 cc. of boiling alcohol. Fine colorless flakes or plate-shaped crystals began to separate at once and the mixture, after cooling, was allowed to stand in the ice box overnight. The crystalline precipitate was filtered off, washed thoroughly with alcohol and ether, and dried in a vacuum desiccator over sulfuric acid. The dry digitonide was extracted with boiling xylene as described by Windaus (12). The digitonin recovered in this manner was used over again until all of the crude cholesterol had been precipitated.

Examination of Material Not Precipitated by Digitonin.

The alcoholic mother liquors from the digitonide were evaporated to dryness under reduced pressure. The residue was extracted with ether; the ethereal solution was filtered and the ether was distilled. The residue was a yellow oil of unpleasant odor which on standing at room temperature gradually deposited a few octahedral crystals; a part separated as long thin flakes and the whole formed a semisolid mass that weighed 3 gm. It is evident therefore that about 18 per cent of the original unsaponifiable matter was not precipitated by digitonin. The material was treated with 60 cc. of hot alcohol which dissolved everything except the yellow octahedral crystals. The latter were identified as sulfur. The clear alcoholic solution was allowed to cool when a part of the dissolved substance separated as an oily layer on the bottom of the flask. The solution was decanted and evaporated to dryness when a yellow oil was obtained. It was impossible to secure any crystals from either of the above mentioned fractions. Both substances gave the Liebermann-Burchard reaction in the same manner as pure cholesterol and in chloroform solution bromine was absorbed. It is probable therefore that the material was closely related to cholesterol although it was not precipitated by digitonin.

The substance was reduced with hydrogen and platinum oxide as described for cholesterol. A considerable amount of hydrogen was absorbed but the reduction product, on evaporation of the solvent, formed a yellow oil that solidified on standing to a thick gummy mass that could not be crystallized from any of the usual organic solvents. In the Liebermann-Burchard reaction it gave a slight greenish yellow fluorescent coloration.

Examination of the Cholesterol that Had Been Precipitated with Digitonin.

The cholesterol which remained in the xylene, after decomposing the digitonide, was obtained by distilling the solvent under reduced pressure and after the last traces of xylene had been removed by distillation with steam. After the solution had cooled the cholesterol was filtered off, washed with water, and dried in a vacuum desiccator over sulfuric acid. The material was a slightly sticky solid that weighed 13.3 gm. The substance was therefore

recovered quantitatively. It was dissolved in 180 cc. of hot alcohol, treated with norit, and recrystallized from alcohol when 5.1 gm. of ball-shaped aggregates of thick plates were obtained that did not resemble cholesterol crystals. The substance sintered at 146° and melted at 149°. A second fraction of impure cholesterol weighing 4.1 gm. was obtained by concentrating the mother liquors.

After the last mentioned crystals had been filtered off, the filtrate was evaporated to dryness. The residue which weighed 3 gm. was a yellow wax-like material. This residue which showed no tendency to crystallize evidently did not contain very much cholesterol because when brominated by the method of Windaus and Hauth (13) no crystals of cholesterol dibromide were obtained. The original unsaponifiable matter contained therefore 6 gm. or about 37 per cent of non-crystalline material, the nature of which is unknown.

Purification of Cholesterol by means of the Dibromide.

The results described above indicate that the unsaponifiable matter obtained from the plasma of normal dog blood contains several substances in addition to cholesterol. A part of this material did not react with digitonin while a portion of non-crystalline substance was precipitated with this reagent along with cholesterol.

Since it appeared to be impossible to secure any pure cholesterol by crystallization, the two fractions of impure crystals described above were united and brominated by the method of Windaus and Hauth (13). The bromination mixture contained an excess of bromine but no hydrobromic acid could be detected. The cholesterol dibromide began to crystallize immediately in colorless needles. After the solution had stood in a freezing mixture for some time the crystals were filtered off, washed with glacial acetic acid, and finally with water. The snow-white substance, after it had been dried in a vacuum desiccator over sulfuric acid, weighed 8.4 gm. When heated in a capillary tube, it softened at 118° and melted to a brown liquid at 122°. The rotation in chloroform solution was −43.53°.

The substance was debrominated by boiling its alcoholic solu-

tion with zinc dust and acetic acid and the reaction product was recrystallized five times from alcohol. The snow-white plates had the characteristic form of cholesterol crystals and after drying in the air weighed 3.2 gm. The substance melted at 150–151° and the melt solidified at 119°; $[\alpha]_D$, −38.11°. From the mother liquors a second fraction of cholesterol was obtained which melted at 150–151°; $[\alpha]_D$, −38.22°. This cholesterol preparation had a slightly higher melting point but the rotation was about 1° lower than the cholesterol isolated from beef blood plasma.

The acetyl derivative was prepared and it was twice recrystallized from alcohol. It separated in burr-shaped aggregates of colorless needles, m.p. 115°; $[\alpha]_D$, −42.56°.

Examination of Commercial Cholesterol.

Cholesterol[1] of unknown origin was used in these experiments. An alcoholic solution containing 100 gm. of cholesterol and 30 gm. of potassium hydroxide was refluxed for 2 hours. One half of the alcohol was distilled off and the cholesterol was precipitated by adding water. The mixture was extracted with ether and the ethereal solution, after washing four times with water, was dried with sodium sulfate and filtered. The filtrate, which was yellowish in color and not quite clear, was shaken with norit and filtered when a colorless solution was obtained. The ether was distilled leaving a snow-white residue which was recrystallized from 700 cc. of alcohol when colorless plates characteristic of cholesterol crystals were obtained. The mother liquor was concentrated and the material that separated was recrystallized from alcohol, giving colorless plates that appeared to be identical with the first lot. The properties of the two fractions were practically identical as shown in Table III.

Acetyl Derivative.

The acetyl derivative was prepared by boiling 50 gm. of the purified cholesterol with 200 cc. of acetic anhydride for 1 hour. After cooling, the crystals were filtered off and washed with cold methyl alcohol. The snow-white product was recrystallized from

[1] Purchased from Eastman Kodak Company.

alcohol when small colorless needles were obtained. The substance melted at 116–117°, solidified at 108°; $[\alpha]_D$, −43.65°.

This preparation was recrystallized ten times and separated into four fractions. The first two mother liquors were concentrated, yielding Fraction 4; the next three mother liquors gave Fraction 3, while Fraction 2 was obtained by concentrating the last five mother liquors. The properties of these fractions are shown in Table IV.

The top fraction was identical in properties with the original acetyl derivative but the other fractions showed slightly lower rotations and melting points.

TABLE III.

Properties of Purified Cholesterol.

M. p.	Solidifying point.	$[\alpha]_D$ in $CHCl_3$.	Water of crystallization.
°C.	°C.		per cent
150–151	120	−39.72°	5.33
150–151	116	−39.63°	5.24

TABLE IV.

Fractionation of Cholesteryl Acetate.

Fraction No.	Weight.	M.p.	$[\alpha]_D$ in $CHCl_3$.
	gm.	°C.	
1	32.7	116	−43.59°
2	10.0	115–116	−43.39°
3	4.4	115–116	−42.92°
4	5.0	114–116	−42.62°

Fraction 4 which possessed the lowest melting point and rotation was again twice recrystallized from alcohol and separated into two fractions. The bottom fraction was obtained by concentrating the mother liquors. It crystallized in colorless needles and melted at 111–112°. The rotation in chloroform solution was −41.12°.

The results indicate a marked difference in the properties between the top fraction in Table IV and the last bottom fraction. The variation in melting point and rotation must be due to some substance having a lower rotation than ordinary cholesterol. It is evident, however, that only a small amount of this substance

could have been present. In order to determine the nature of the contaminating substance it will be necessary to fractionate larger quantities of cholesterol. It is not improbable that some derivative other than the acetate and that a solvent better than alcohol could be found that would cause a more effective separation, but lack of time has prevented further investigation along these lines.

In conclusion the author takes pleasure in acknowledging his indebtedness to Dr. W. R. Bloor of the University of Rochester, for generously supplying crude cholesterol.

SUMMARY.

Attention is called to the fact that cholesterol preparations obtained from different sources show slight differences in physical properties.

When apparently pure cholesteryl acetate is fractionally recrystallized from ethyl alcohol, it is possible to separate a bottom fraction that possesses a much lower melting point and a lower optical rotation than the top fraction.

If cholesterol is formed from plant sterols a number of different as well as isomeric cholesterols might be expected to occur in animal fats corresponding to the various phytosterols contained in the plant material which serves as food.

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A NOTE ON THE NATURE OF THE PICRATE OBTAINED FROM NORMAL URINE BY THE METHOD OF FINDLAY AND SHARPE.

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As the preliminary step to a study on the metabolism of guanidine and its derivatives, it has become necessary to investigate the possible urinary excretion of the guanidine bases. Based on the publications of Kutscher, Achelis, Engeland, Smorodinzev, and others, text-books now state that methylguanidine, accompanied by asymmetric dimethylguanidine, is a normal constituent of blood, muscular tissue, and urine. The truth of this statement cannot be unreservedly accepted, since the work of Ewins (1) and Baumann and Ingvaldsen (2) has thrown doubt on the methods used for the isolation of these bases. These methods, or slight modifications of them, were also used by Koch (3, 4) and by Paton and Findlay (5, 6) and Burns and Sharpe (7) in the work which led to the theory advanced by Paton connecting the guanidine bases with the function of the parathyroids. In 1920 Findlay and Sharpe (8) elaborated a procedure free from the objections advanced to the previous methods, and this, since slightly modified by Sharpe (9) on account of Greenwald's criticism (10), has been used in all subsequent investigations by Paton and his collaborators. It has also been applied by Major (11, 12) to normal and various pathological urines, in support of his theory that essential hypertension is due to deficient guanidine excretion.

As modified by Sharpe, the method consists in the treatment of 250 cc. of urine successively with tannic acid, baryta, sulfuric acid, and barium carbonate, the precipitates being filtered off as produced. The final filtrate is evaporated to a syrup, extracted with 60 cc. of absolute alcohol, the filtered extract again evaporated,

and the residue extracted with 20 cc. of absolute alcohol. After removal of the solvent the residue is dissolved in 10 cc. of water and treated with 10 cc. of a saturated solution of picric acid. It is claimed that the picrate crystals which separate represent the guanidine content of the urine.

Using this procedure, crystals of dimethylguanidine picrate have been reported,[1] the only means of identification recorded being the appearance, melting point, and nitrogen content. While the melting point figures agree with that of dimethylguanidine picrate which is variously recorded as 225°C. (Greenwald (10)), 227°C. (Werner and Bell (14)), and 229–230°C. (Phillips and Clarke (15)), the figures for the nitrogen percentage do not show this agreement (Findlay and Sharpe (8), 29.4 and 28.7; Nattrass and Sharpe (16), 24.0; Major (12), 26.0; calculated value, 26.6). No attempt appears to have been made to obtain and identify the base itself or any other of its salts. Since this did not appear to be a difficult procedure, several normal urines have been treated according to Findlay and Sharpe's directions, as modified by Sharpe, and the results are now recorded.

EXPERIMENTAL.

In all, four samples of normal male human urine were examined; one a mixed sample, and the others, the 24 hours excretions of three different individuals. These were all collected under toluene, and were worked up within 24 hours. In order to follow Sharpe's procedure in every particular, in the case of two urines, 250 cc. were taken and worked up separately from the remainder. The various samples were then treated as previously described except that in the cases of Urines B, C, and D, following the original procedure after addition of barium carbonate, hydrochloric acid was added until the liquor was acid to litmus but not to Congo red. This was in order to guard against the possibility of barium hydroxide being present in the carbonate, and the consequent alkalinity of the liquor during evaporation, tending to decomposi-

[1] In a recent communication, Paton and Sharpe (13) report an increase in monomethylguanidine in the blood of dogs after parathyroidectomy. The method used is stated to be the picrate method evolved for urine; no details are given, and the methylguanidine picrate is identified solely by its appearance and melting point.

tion and loss of the volatile bases. Urine Samples A, C1, and C2, were evaporated on the open water bath, and the remainder, under diminished pressure. In all cases, picrate crystals were obtained of the same appearance and crystalline structure, and with melting points which ranged from 223–232°C. As far as microscopic examination went, the crystals appeared to be perfectly homogeneous with no indication of the presence of any other picrate. After these were filtered off, excess picric acid was added, and in each case a further crop of crystals was obtained, which proved to be homogeneous and merely a second crop of the same picrate. The details are found in Table I.

TABLE I.

Sample.	Description.	Volume.	Yield of picrate.		Melting point.		Total yield.
			1st crop.	2nd crop.	1st crop.	2nd crop.	
		cc.	*mg.*	*mg.*	*°C.*	*°C.*	*mg.*
A	Mixed.	1450			233		700
B1	24 hour sample.	250	149	121	229	224	1168
B2		1310	525	373	225	227	
C1	24 " "	250	164	100	230	229	989
C2		600	396	329	231	232	
D	24 " "	870	547	448	224	228	995

Although the total yield is given in Table I, this would not be the yield according to Sharpe's procedure, which calls for the single addition of a definite amount of picric acid. Consequently, for purposes of comparison with some of the yields given in the literature, only the first crops of picrate crystals form the basis of calculation. These figures are shown in Table II.

Examination of Picrate Crystals.

The picrate on examination was found to consist of long irregular rectangular plates of a dark yellow to chocolate-brown color. It was recrystallized from hot water (a little charcoal being added to remove the urinary pigment), and was obtained

in the form of long needles of a golden yellow color, quite unlike the crystalline form of dimethylguanidine picrate. The melting points after recrystallization were in every case higher, the values ranging from 235–241°C., and the melting being invariably accompanied

TABLE II.

Author.	Description of case.	Yield of dimethyl-guanidine.		Corresponding amount of picrate.	
		mg. per liter	*mg. per day*	*mg. per liter*	*mg. per day*
White.					
Sample B.	Normal.			430	674
" C.	"			660	560
" D.	"			630	547
Findlay and Sharp.	Tetany.	190	175	690	635
		90	83	327	301
Nattrass and Sharpe.	"		305*		1488
Major.					
Sample 1.	Normal.		130		472
	"		100		363
" 2.	Leukemia.		140		508
" 3.	Tuberculosis of hip.		186		675
" 4.	Essential hypertension.		60		218
	" "		43		146
" 5.	Chronic nephritis.		200		726
" 6.	Essential hypertension.				
	Case 1†	210	275	762	998
	" 1‡	186	410	675	2288
	" 2†	170	60	617	218
	" 2‡	170	320	617	1162

* Value expressed as mg. of guanidine.

† Value before treatment with digitalis and diuretin.

‡ Value when blood pressure reached its lowest point after treatment.

by the evolution of gas. The total yield of picrate obtained from urine Sample D (see Table I) was recrystallized several times from water and from alcohol, and was then found to melt at 244°C., with further evolution of gas at about 260°C. It gave a positive Jaffé test for creatinine. This behavior is similar to that of

creatinine potassium picrate, which Greenwald and Gross (17) reported melted at 247°C. with further evolution of gas at about 270°C. Accordingly some creatinine potassium picrate was prepared by the addition of excess of a saturated solution of picric acid to an equimolecular mixture of creatinine and potassium chloride. After recrystallization from hot water this melted at 245°C. with evolution of gas at about 265°C.; mixed with the picrate from urine, the values were 244° and 258°C., respectively. A mixture of the urine picrate with that of dimethylguanidine melted with decomposition at 206–208°C. These indications pointed to creatinine potassium picrate as the substance obtained from urine, and this was confirmed by analysis. Picric acid was estimated by the nitron method of Busch and Blume (18); creatinine by Folin's colorimetric procedure; and nitrogen by the micro-Kjeldahl method as modified for nitro compounds by Elek and Sobotka (19).

24.3 mg. picrate gave 18.3 mg. picric acid, or 75.3 per cent.
11.2 " " contained 2.12 mg. creatinine, or 18.9 per cent.
8.0 " " gave 1.67 mg. nitrogen, or 20.9 per cent.

Calculated for creatinine picrate: picric acid, 67.0 per cent; creatinine, 33.0 per cent; nitrogen, 24.6 per cent.

Calculated for creatinine potassium picrate: picric acid, 75.2 per cent; creatinine, 18.7 per cent; nitrogen, 20.7 per cent.

The filtrate from nitron picrate gave a positive test for potassium. A nitrogen estimation on a sample of the crude picrate gave a value of 21.3 per cent, indicating the presence of a small amount of impurity (probably creatinine picrate) which had almost all been removed by recrystallization.

DISCUSSION.

Using Findlay and Sharpe's procedure as modified by the latter, a picrate has been obtained whose melting point is similar to those reported in the literature, but which is not dimethylguanidine picrate. This may not be the compound obtained by Sharpe and his colleagues, since it is difficult to see how the high nitrogen values reported by them could have been obtained from creatinine potassium picrate, while a Jaffé test would at once have shown the presence of creatinine, and further, they were investigating

pathological urines, whereas the data here refer to normal urines. It seems probable, however, that this is the picrate obtained by Major, since his theory is based upon a diminution, in cases of essential hypertension, of the amount of picrate obtained from normal urine. It is true that Major claims to have obtained a nitrogen value of 26 per cent which he erroneously states is correct for dimethylguanidine (the calculated value is 26.6 per cent). He also states that a mixture of his picrate with that of dimethylguanidine gave a "sharp and uniform melting point," although previously (11) he admits that his picrate is not free from creatinine, and that to get his dimethylguanidine values, creatinine was estimated by Folin's method and the calculated value of creatinine picrate subtracted from the total. If this were done with creatinine potassium picrate, there would remain almost half the total picrate unaccounted for. The melting point obtained by him (225°C.) is remarkably high for a dimethylguanidine picrate admittedly contaminated with creatinine picrate. It is evident that Major has not offered sufficient proof of the identity of the picrate obtained by him, and that it is probably creatinine potassium picrate.

A critical examination of the charts of two hypertension cases which Major has published (12), reveals the fact that whereas the daily excretion of "dimethylguanidine" increases with fall of blood pressure, so does the volume of urine (digitalis and diuretin were administered). Consequently, the amount of "dimethylguanidine" excreted per liter of urine does *not* show any increase (see Table II). It could therefore be argued that the hypertension might equally well be connected with the retention of any of the urinary constituents.

Greenwald (20) has recently criticized Sharpe's modified method on the grounds that no adequate provision is made for removal from the urine of creatinine and ammonium salts, and that, while his method is apparently based upon the slight solubility of guanidine picrate, he seems to have lost sight of the fact that the picrates of mono- and dimethylguanidine are considerably more soluble than that of guanidine. The solubility factor being of prime importance, this discrepancy had also been noticed during the course of this investigation, and the solubilities of various picrates in pure water compared, under the same conditions, at

room temperature. These are shown in Table III, together with Greenwald's figures for the solubility at 20°C., and those of Medes (21) at 21°C. The method I have used has been recently described elsewhere (22).

It is obvious that Greenwald's contention is correct—Sharpe's method is not applicable to the determination of guanidines in urine. The solubilities of the picrates of creatinine, mono-, and dimethylguanidine are such as to preclude the possibility of either of the guanidine picrates being obtained uncontaminated with creatinine picrate. Further, creatinine potassium picrate, if present in any reasonable amount, is bound to crystallize out before, or together with, either of the guanidine picrates.

TABLE III.

Picrate.	Melting point found (uncorrected).	Temperature.	Solubility in mg. per 100 cc.		
			White.	Greenwald (at 20°C.).	Medes (at 21°C.).
	°C.	°C.			
Creatinine	212–214	20.0	179		182
Creatinine-potassium	245	21.8	157		
(From urine.)	244	19.7	152		
Guanidine	317–320	20.0	63		61
Methylguanidine	197	20.1	217	178	
α,α-Dimethylguanidine	225	21.0	170	162	

The extent to which this double picrate may be formed hinges on the amount of potassium present in the final extract. Solubility experiments with Kahlbaum's pre-war "Zur Analysis" potassium chloride (previously dried at 105°C.) showed that at 20°C. 100 cc. of absolute alcohol dissolve 27 mg., and 100 cc. of 95 per cent alcohol dissolve 87 mg. Since the final extract from 250 cc. of urine is made with 20 cc. of absolute alcohol, this could contain 5.4 mg. of potassium chloride, yielding 44 mg. of the double picrate, which is more than sufficient to saturate the picric acid solution subsequently used. It is certain that under the conditions of the procedure some potassium will be dissolved and that the more water present during the alcoholic extraction, the more potassium will go into solution. To reduce this to a

minimum the presence of water in the final alcoholic extract must be excluded; this demands a more rigorous procedure than the two alternating extractions and evaporations recommended by Sharpe.

The experiments reported here have afforded no indication of the presence in normal urine of any guanidine bases. The method, however, is faulty and the question must remain *sub judice* until a new method is devised which will estimate small quantities of the guanidine bases in presence of creatinine and other urinary constituents. An attempt to develop such a method is at present in progress.

SUMMARY.

By the procedure advocated by Findlay and Sharpe for the estimation of guanidine bases in urine, no guanidine bases have been found in four samples of normal human urine. A considerable yield of creatinine potassium picrate has however been obtained, and it is regarded as probable that this is the substance obtained by Major and assumed by him to be dimethylguanidine picrate.

It follows that no satisfactory chemical evidence exists connecting essential hypertension with retention of guanidine bases.

Greenwald's contention that Sharpe's method cannot be used for the estimation of guanidine bases in urine, is upheld.

The author wishes to express his indebtedness to Professor A. T. Cameron for his constant interest and advice during the course of this work.

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THE PROTEIN VALUE IN NUTRITION OF BEEF LIVER, BEEF HEART, AND BEEF KIDNEY.

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It is a singular fact that in the preparation of animal carcasses for human consumption, as in the preparation of the cereal grains, the portions of the product in least demand have been found to possess the greatest dietetic value with reference to certain nutrients. The superiority of many of the visceral organs of animals over the muscle meats as sources of vitamins is analogous to the superiority of the germ and outer layers of the cereal grains over the endosperm. In the latter case, the superiority is known to extend to the proteins also, while in the former case, McCollum, Simmonds, and Parsons (1) have reported observations upon rats that have been interpreted to mean that the proteins of beef liver, and particularly of beef kidney, are superior in nutritive value to the proteins of beef muscle. In fact, kidney proteins are placed higher by these investigators than those of milk or of any animal tissues yet studied. The existence of several uncertainties (2) in the evaluation of the type of experimental observations upon which this conclusion was based would appear to detract from its force and render advisable the securing of similar data by the use of experimental methods more nearly quantitative in character and restricted as nearly as possible to one function, such as growth.

Nitrogen metabolism studies, according to the routine adopted in this laboratory (3), were therefore made upon a series of twenty-five rats to determine the biological value of the nitrogen of beef heart, beef liver, and beef kidney. While the investigation was still in progress, a paper by Hoagland and Snider (4) appeared, reporting the results of experiments concerned with the nutritive

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value of the proteins (N × 6.25) of muscle, liver, heart, and kidney from cattle, sheep, and hogs. The method used was that developed by Osborne, Mendel, and Ferry (5), in which the gain of growing rats per gm. of protein consumed under certain dietary conditions is taken as the measure of the excellence of the protein fed. From a large number of experimental results exhibiting considerable variability, only partially explainable by differences in food consumption, Hoagland and Snider conclude, somewhat

TABLE I.

Composition of Rations.

Ingredients.	4 per cent egg protein.	8 per cent heart protein.	8 per cent kidney protein.	8 per cent liver protein.	16 per cent liver protein.
Whole dried egg*	5.02				
Dried beef heart*		10.09			
" " kidney*			10.69		
" " liver*				12.51	25.02
Salts	4	4	4	4	4
Butter fat	10	10	10	10	10
Sucrose	10	10	10	10	10
Cellu flour†	4	4	4	4	4
NaCl	1	1	1	1	1
Starch	65.98	60.91	60.31	58.49	45.98
Total	100	100	100	100	100

*These products were freed from all visible fat and dried at a low temperature. The dried products contained the following per cents of nitrogen: egg, 12.56; beef heart, 12.52; beef liver, 10.23; and beef kidney, 12.05.

†A product obtained from the Chicago Dietetic Supply House containing 37.8 per cent crude fiber and 0.015 per cent nitrogen.

non-commitally, that "As regards the probable relative nutritive values of voluntary muscle, heart, liver, and kidney from the ox, sheep, and hog, as sources of protein in the human dietary, it seems likely that when the diet contains an adequate amount of protein these tissues will have approximately the same value for maintenance and growth."

In the experiments to be reported in this paper, the rations to be compared contained approximately 8 per cent of protein (N × 6.25) from each of the three different animal products, ex-

TABLE II.

Summary of Biological Values.

Rat No.	8 per cent heart protein.	8 per cent kidney protein.	8 per cent liver protein.	16 per cent liver protein.
371	65	73	76	
372	64	74	80	
373	70	72	76	
374	75	86	83	
375	67	65	77	
Average.....	68	74	78	
381	77	63	67	
382	89	90	79	
383	78	83	74	
384	92	86	72	
385	85	80	81	
Average.....	84	81	75	
421	79	85	71	
422	79	71	77	
423	75	76	74	
424	75	80	77	
425	73	76	74	
Average.....	76	78	75	
431	71	75	74	
432	67	70	76	
433	67	73	73	
434	69	75	71	
435	73	74	73	
Average.....	70	74	74	
391			80	57
392			83	66
393			80	57
394			82	58
395			84	51
Average.....			82	58
Grand average.....	74	77	77	58

TABLE III.

Nitrogen Metabolism Data and Biological Values.

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
Period I. Egg ration, containing 0.69 per cent N.							
371	121	128	8.0		19	22	
372	130	136	7.9		18	18	
373	134	142	8.0		17	16	
374	120	122	7.5		17	21	
375	100	99	5.8		16	15	
Period II. Liver ration, containing 1.28 per cent N.							
371	133	144	8.0	102	22	48	76
372	142	151	7.4	95	19	39	80
373	150	162	8.0	103	18	44	76
374	130	137	7.3	94	21	39	83
375	107	110	5.5	70	13	33	77
Period III. Kidney ration, containing 1.31 per cent N.							
371	147	153	8.1	107	22	53	73
372	157	167	9.0	118	19	54	74
373	172	180	9.0	118	20	57	72
374	143	153	8.0	105	16	40	86
375	115	121	8.0	105	19	56	65
Period IV. Heart ration, containing 1.30 per cent N.							
371	155	160	7.8	102	19	61	65
372	176	180	9.0	117	20	67	64
373	192	197	9.0	117	20	63	70
374	161	166	8.0	104	17	53	75
375	131	136	7.7	100	20	55	67
Period V. Egg ration, containing 0.67 per cent N.							
371	156	163	8.5		21	25	
372	183	178	6.9		19	26	
373	198	198	9.4		20	30	
374	169	168	7.7		19	29	
375	129	131	7.0		17	23	

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
Period I. Egg ration, containing 0.69 per cent N.							
381	111	116	7.3		17	17	
382	138	143	8.0		17	19	
383	132	134	7.1		16	19	
384	122	125	8.0		18	19	
385	109	112	7.2		15	15	
Period II. Heart ration, containing 1.30 per cent N.							
381	121	127	6.8	89	19	41	77
382	151	163	8.0	104	16	35	89
383	144	157	8.0	104	18	47	78
384	132	145	8.0	104	16	32	92
385	121	134	8.0	104	17	37	85
Period III. Kidney ration, containing 1.31 per cent N.							
381	129	129	8.0	105	22	63	63
382	171	178	9.0	118	18	40	90
383	165	172	9.0	118	19	48	83
384	156	161	9.0	118	17	46	86
385	142	147	9.0	118	20	52	80
Period IV. Liver ration, containing 1.28 per cent N.							
381	138	141	8.3	106	28	63	67
382	186	194	10.0	128	24	60	79
383	179	187	10.0	128	26	64	74
384	167	174	10.0	128	24	70	72
385	155	167	10.0	128	26	61	81
Period V. Egg ration, containing 0.67 per cent N.							
381	140	130	4.2		13	31	
382	195	189	8.2		20	36	
383	190	176	5.1		14	33	
384	174	163	5.2		14	37	
385	160	144	3.9		11	40	

TABLE IV.

Nitrogen Metabolism Data and Biological Values.

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
Period I. Egg ration, containing 0.66 per cent N.							
421	59	66	8.0		9	16	
422	55	63	7.9		9	15	
423	55	62	7.8		8	18	
424	53	59	7.3		8	12	
425	46	52	6.4		8	8	
Period II. Heart ration, containing 1.27 per cent N.							
421	75	87	8.0	101	10	42	79
422	74	85	8.0	101	10	41	79
423	71	81	8.0	101	10	48	75
424	71	83	8.0	101	10	42	75
425	64	78	8.0	101	9	40	73
Period III. Kidney ration, containing 1.27 per cent N.							
421	97	117	9.0	114	13	43	85
422	95	112	9.0	114	14	58	71
423	92	109	9.0	114	12	56	76
424	92	112	9.0	114	13	45	80
425	87	108	9.0	114	12	46	76
Period IV. Liver ration, containing 1.25 per cent N.							
421	123	133	9.0	113	17	61	71
422	121	134	9.0	113	15	56	77
423	117	126	9.0	113	16	63	74
424	120	133	9.0	113	15	53	77
425	119	130	9.0	113	15	55	74
Period V. Egg ration, containing 0.66 per cent N.							
421	133	120	5.1		7	29	
422	138	127	6.1		9	32	
423	128	120	5.1		8	34	
424	135	130	6.8		8	30	
425	134	125	5.2		7	28	

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
Period I. Egg ration, containing 0.66 per cent N.							
431	59	65	8.0		8	17	
432	58	65	8.0		10	15	
433	57	62	7.5		10	17	
434	43	50	6.3		8	12	
435	48	55	6.6		7	12	
Period II. Liver ration, containing 1.25 per cent N.							
431	75	86	8.0	100	12	43	74
432	74	85	8.0	100	13	42	76
433	71	80	8.0	100	12	45	73
434	60	71	8.0	100	12	44	71
435	65	76	8.0	100	12	41	73
Period III. Kidney ration, containing 1.27 per cent N.							
431	97	115	9.0	114	12	49	75
432	95	111	9.0	114	13	55	70
433	92	110	9.0	114	14	51	73
434	80	97	9.0	114	13	48	75
435	86	107	9.0	114	13	47	74
Period IV. Heart ration, containing 1.27 per cent N.							
431	123	136	9.0	114	13	53	71
432	117	127	9.0	114	15	60	67
433	117	126	8.6	109	12	57	67
434	107	118	9.0	114	10	59	69
435	118	128	8.4	107	10	50	73
Period V. Egg ration, containing 0.66 per cent N.							
431	139	144	9.0		10	18	
432	129	134	9.0		10	23	
433	128	137	9.0		11	20	
434	122	129	9.0		11	24	
435	131	140	9.0		12	21	

cept that one group of rats was fed liver protein at a 16 per cent, as well as an 8 per cent, level. The composition of the rations is given in Table I. Each rat received per day, besides its allotment of ration, 25 mg. of yeast vitamin (Harris), containing from 2 to 3 mg. of nitrogen, and 1 or 2 drops of cod liver oil.

The results of the nitrogen balance data are used in the calculation of the biological value by a method fully explained and de-

TABLE V.

Nitrogen Metabolism Data and Biological Values.

Rat No.	Initial weight.	Final weight.	Food intake.	N intake.	Fecal N.	Urinary N.	Biological value.
	gm.	*gm.*	*gm.*	*mg.*	*mg.*	*mg.*	*per cent*
Period I. Egg ration, containing 0.65 per cent N.							
391	59	62	6.4		14	10	
392	61	65	6.4		13	11	
393	60	62	6.5		14	11	
394	59	62	6.3		14	10	
395	65	64	5.4		12	12	
Period II. Liver ration, containing 1.28 per cent N.							
391	72	86	8.0	103	19	35	80
392	73	86	8.0	103	15	33	83
393	69	84	8.0	103	20	34	80
394	69	81	7.4	95	20	30	82
395	72	86	7.7	99	22	31	84
Period III. Liver ration, containing 2.57 per cent N.							
391	99	115	8.0	206	25	105	57
392	100	118	8.0	206	23	91	66
393	98	112	8.0	206	27	106	57
394	92	110	8.0	206	24	105	58
395	96	109	8.0	206	26	120	51
Period IV. Egg ration, containing 0.67 per cent N.							
391	119	115	6.4		17	24	
392	123	114	5.6		14	30	
393	115	112	6.4		16	24	
394	117	112	5.9		14	26	
395	115	108	5.6		15	26	

fended elsewhere (3). The biological value is taken to measure the percentage of absorbed nitrogen that is used in the body for maintenance and growth. The detailed data of these studies are given in Tables III, IV, and V, while the biological values are summarized in Table II.

Except for the high values obtained for heart protein with Rats 381 to 385, the results are as consistent among themselves as is usual in this indirect determination. The average biologi-

cal values obtained at the 8 per cent level were 74 for beef heart, and 77 for beef kidney and beef liver. The difference is probably of no significance and the conclusion seems justified that the nitrogen of these three tissues is utilizable in anabolism to approximately the same extent. Furthermore, the average biological values obtained are equal to or slightly higher than the values obtained in other experiments for muscle meats; *i.e.*, 74 for pork (6) and 69 for beef (7). On the other hand, they are less than the values of 83 to 86 consistently obtained in this laboratory for milk proteins (7, 8).

At the 16 per cent level, the crude protein of liver was found to possess a distinctly smaller biological value, averaging 58, than at the 8 per cent level. This is but another confirmation of the conclusion deduced from earlier experiments (9) to the effect that the utilization of protein (nitrogen) in anabolism varies inversely with the level at which it is fed, as measured by the percentage of nitrogen in the ration.

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A BIOCHEMICAL STUDY OF TOOTH GROWTH.

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A knowledge of the chemical differentiation of normal teeth during growth is a prerequisite for the study of the influence of experimental procedures on tooth composition, for the study of the changes induced in teeth by disease, and for the study of systemic chemical development.

Many scattered reports are to be found in the literature concerning the composition of teeth, but these are isolated studies unbacked by systematic controlled observation. Hence a study was made of the age changes in tooth composition under adequately controlled conditions of diet, environment, age, and sex. The albino rat was chosen as the experimental animal because it lends itself to such control. The investigation was restricted to a determination of the water, ash, organic matter, calcium, phosphorus, and magnesium of the upper and lower incisors of the male animal at 23, 30, 50, 65, 75, 100, and 150 days of age.

The data to be reported were obtained from the whole depulped tooth. The upper and lower incisors were analyzed separately. The figures given in the tables are the means of five sets of duplicate analyses reduced to the value for the single tooth. The number of teeth in each set and consequently the number of animals used varied with the age of the rats. More teeth were required to give an adequate amount of ash for analysis from the younger than from the older rats.

The rats came from the Experimental Colony stock of The Wistar Institute, and had been raised on the varied diet and in the environment described by Greenman and Duhring (1). The methods used for the determination of the water, organic matter, and ash were those described by Hammett (2). Calcium was determined according to the method of Kramer and Tisdall

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(3), magnesium by the method of Hammett and Adams (4), and phosphorus according to Bell and Doisy (5).

In Table I are given the mean observed values for the body weight of the several groups of rats, and the weight, ash, organic matter, and water of the teeth.

TABLE I.

Mean Observed Body and Tooth Weight, and Ash, Organic Matter, and Water of Upper and Lower Incisor Teeth at Stated Ages.

Age.	Body weight.	Weight.		Ash.		Organic matter.		Water.	
		Upper.	Lower.	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.
days	*gm.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
23	30.5	13.9	15.2	8.5	9.5	2.8	3.1	2.6	2.6
30	47.4	21.0	22.8	13.0	14.5	4.0	4.3	3.9	4.0
50	96.7	38.5	37.8	24.6	24.7	7.3	7.0	6.6	6.1
65	143.1	49.5	48.7	32.3	32.6	9.3	9.1	7.9	7.0
75	159.1	56.4	56.6	36.5	37.6	10.6	9.8	9.3	9.1
100	196.4	65.8	63.2	43.9	42.6	12.8	12.5	9.1	8.1
150	228.6	82.6	84.2	56.7	57.6	14.6	15.9	11.4	10.7

TABLE II.

Mean Per Cent Ash, Water, and Organic Matter, Calcium, Phosphorus, and Magnesium of Upper and Lower Incisor Teeth at Stated Ages.

Age.	Ash.		Organic matter.		Water.		Calcium.		Magnesium		Phosphorus.	
	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
23	60.9	62.4	20.3	20.2	18.8	17.4	21.8	22.5	1.16	0.99	12.7	12.9
30	62.2	63.6	19.1	19.1	18.7	17.4	22.6	23.4	1.22	0.99	12.8	12.9
50	64.0	65.4	19.1	18.8	16.9	15.8	22.9	23.8	1.38	1.14	13.2	13.1
65	65.0	66.9	18.9	18.7	16.0	14.4	23.0	24.1	1.51	1.26	13.8	14.6
75	64.7	66.6	18.7	17.2	16.5	16.2	22.7	23.7	1.46	1.15	12.7	12.8
100	66.7	67.3	19.5	19.9	13.8	12.8	23.5	24.4	1.56	1.26	13.7	14.1
150	68.6	68.6	17.7	18.9	13.7	12.5	24.1	24.5	1.51	1.25	13.4	13.7

Two points of interest are found. The first is the fact that the lower incisor is heavier than the upper. This finding is consistent with the report in Donaldson's book on the rat (6) that the lower incisor is longer than the upper. The weight difference is

largely due to the difference in ash. This is consistent with Marshall's (7) observation that the enamel of the lower incisor is thicker than that of the upper in this species.

The second point of interest is the fact that there is absolutely less water by weight in the teeth at 100 than at 75 days of age. This loss of water is similar to that noted by Hammett (8) in the long bones. The absolute amounts of ash, organic matter, and water, with this exception, increase with age.

Table II contains the percentage composition of the teeth at the various ages. From the table it is seen that the lower incisor has a higher percentage as well as a greater absolute amount of ash than the upper tooth. The percentage ash increases with age in both teeth while that of water decreases. Moreover the percentage water of the lower incisor is less than that of the upper. Since the differences in organic matter per cent are inconsistent, and the age changes irregular, it is clear that the chief basis of the tooth composition difference lies in the difference in reciprocal relation between ash and water. The data indicate that in the teeth as in the bones (8) ash displaces water during growth.

Evidence of pubertal influence on the phase of metabolism concerned in the water content of the teeth is shown in Table II. The progressive decrease in the percentage of water is interrupted at 75 days of age (the height of the pubertal adjustment), to be resumed again in the later groups. This is further evidence, obtained from a different set of rats, supporting the concept derived from the observations of Hatai (9) on blood serum, and of Hammett on the bones (8) and central nervous system (10), that puberty conditions an upset in the water exchange of the organism. It is due to the surge in gonadal incretory activity taking place at this time.

If the figures in Table I together with like data for the body length be plotted on semilogarithmic paper (chart not given) certain relations of interest are brought out.

The course of increase of the incisors in weight is quite like that of the body in weight but dissimilar from that of the body in length. The same is true of the three constituents studied. Nevertheless the percentage rate of growth of the teeth tends to fall away progressively from that of the body. This is what is to be expected from the known differences in compositional make-up and their relation to the metabolic cycle.

The percentage rate of increase in ash is greater than that of the teeth as a whole, while the rate of increase in water is less, with the organic matter running an intermediate course.

The upper incisors do not consistently differ from the lower with respect to the percentage rate of growth in weight, or in increments of ash, organic matter, and water. This practical identity in percentage growth rate indicates that the rate of the chemical processes concerned in the formation of the two sets of teeth is also of the same order. Hence, since the lower incisor is more slender because of its anatomical relations, it grows more rapidly in length than the upper. The anatomical relations also determine the greater length of this tooth. These

TABLE III.

Mean Observed Amount of Calcium, Magnesium, and Phosphorus in Incisor Teeth on Age.

Age.	Calcium.		Magnesium.		Phosphorus.	
	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.
days	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
23	3.02	3.42	0.16	0.15	1.76	1.96
30	4.75	5.31	0.26	0.23	2.67	2.94
50	8.77	8.96	0.53	0.43	5.09	4.93
65	11.39	11.75	0.75	0.61	6.85	6.85
75	12.77	13.40	0.82	0.65	7.17	7.23
100	15.46	15.39	1.04	0.80	9.06	8.92
150	19.89	20.59	1.25	1.05	11.09	11.58

factors combine to produce a tooth of higher ash percentage, and hence greater weight, since ash deposition is the characteristic end-result of tooth formation.

Turning now to the age changes in calcium, magnesium, and phosphorus, in Table III are given the mean observed amounts of these elements.

From these figures it is seen that the age and intertooth differences in calcium and phosphorus are quite like those exhibited by the ash. Magnesium, on the other hand, is less in the lower than in the upper incisor. Therefore, magnesium, as an ash constituent, differs from the other two.

Table II gives the percentage of calcium, magnesium, and phos-

phorus in the teeth on age. It is seen that the orderly progression of increase in percentage with age is interrupted by the pubertal adjustment. The two chief ash components, calcium and phosphorus, exhibit the same general direction of difference in the teeth as does the ash. Magnesium, on the other hand, shows the reverse relation; *i.e.*, a lesser percentage in the lower than in the upper incisor at all ages.

If the values given in Table I be charted on semilogarithmic paper (chart not given) it is found that phosphorus and calcium run parallel courses, which are the same as that for ash, while that for magnesium is distinctive and higher. This indicates that

TABLE IV.

Percentage of Calcium, Magnesium, and Phosphorus of the Ash of Incisor Teeth on Age.

Age.	Calcium.		Magnesium.		Phosphorus.	
	Upper.	Lower.	Upper.	Lower.	Upper.	Lower.
days	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
23	35.8	36.0	1.91	1.59	20.8	20.6
30	36.4	36.7	1.96	1.56	20.5	20.3
50	35.8	36.5	2.15	1.74	20.7	20.1
65	35.3	36.0	2.32	1.88	21.2	21.0
75	35.0	35.6	2.25	1.73	19.7	19.2
100	35.2	36.2	2.35	1.88	20.6	21 0
150	35.1	35 5	2.20	1.82	19.6	20 0

the percentage rate of increment in ash of the teeth is largely determined by the rate of formation or deposition of calcium phosphorus complexes. Since the percentage rate of increment in magnesium differs from that of calcium, phosphorus, and ash, and since in fact, magnesium differs from these in the other respects noted in previous paragraphs, it is evident that in the development of the teeth as in that of the bones (11), magnesium participation follows a course different from that of calcium. This essential separateness needs further investigation before interpretation is possible.

Like the ash, so do calcium and phosphorus show no tooth difference in percentage rate of increment.

The percentage rate of increment of magnesium in the upper

incisor tends to increase away from that of the lower as the age increases. Here again magnesium differs from calcium and phosphorus.

As with the ash, organic matter, and water, so with the calcium, magnesium, and phosphorus, the percentage rate of increment decreases as the animal grows older. This decrease is

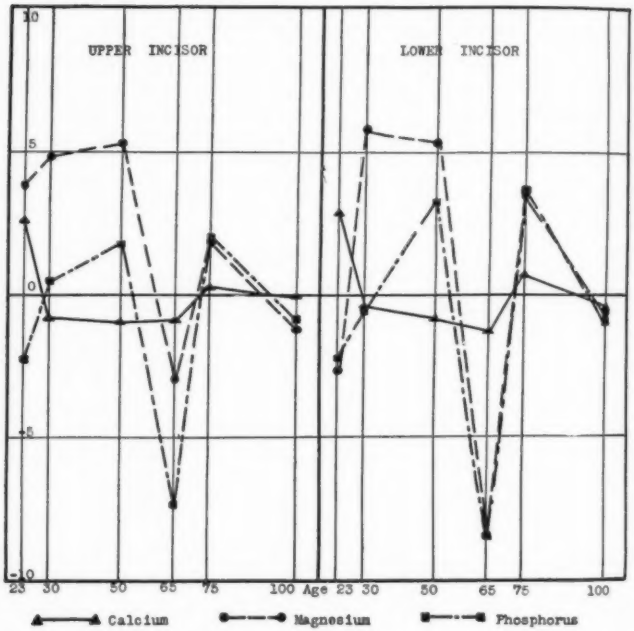

CHART 1. The rate of change in percentage of calcium, magnesium, and phosphorus in the ash of the incisor teeth.

sharp and rapid up to the time of the pubertal adjustment (65 days of age). From then on it is gradual and slow. The teeth thus resemble the bones (8) in the male albino rat, both in the time of onset of the beginning of the stabilization of the rate of chemical differentiation to the level characteristic of the adult growing organism, and in the character of the change which is brought about by the surge in gonadal incretory activity of

puberty. This is further evidence that the metabolic adjustments of puberty are wide-spread and include profound changes in differential chemical development as pointed out by Hammett (8).

With regard to the age changes in the percentage of calcium, magnesium, and phosphorus in the ash, the significant figures are given in Table IV.

It is seen that the percentage of calcium in the ash of the lower incisor is slightly but consistently greater than that in the upper. The percentage of magnesium, on the other hand, is less in the lower than in the upper tooth. The differences in percentage of phosphorus are inconstant in direction. Calcium and phosphorus exhibit no consistent directional shift in percentage value on age, magnesium tends definitely to increase in this respect. The differences, while uninterpretable, are significant in that they again show magnesium to be an ash constituent of different history from that of calcium.

The teeth thus differ from the bones (12) in that the percentage of calcium and phosphorus in the ash changes with age in the latter, but not in the former. On the other hand the teeth are like the bones in that ossification and calcification of the structure as a whole progressively increase during growth.

A pubertal influence on ash composition is shown by the fact that the percentage values of all three constituents at 75 days of age are lower than those which exist at 65 or 100 days. While this difference seems slight in the tables, it is brought out more clearly if the percentage rates of change in the percentage values be computed. These values are plotted in Chart 1, which shows the point.

CONCLUSIONS.

The incisor teeth of the male albino rat increase in weight, ash, organic matter, water, calcium, phosphorus, and magnesium during the growth period from 23 to 150 days of age. The lower incisor is heavier than the upper. This weight difference is largely determined by the ash.

The percentage of ash and its constituents (calcium, magnesium and phosphorus) in the whole tooth increases with age. That of water decreases. Organic matter runs an indeterminate course. Ash displaces water during tooth growth.

The percentage of ash, calcium, and phosphorus is greater in the lower than in the upper incisor.

The calcium and phosphorus percentage of the ash shows no progressive change with age. The percentage of magnesium tends to increase with age. For this and other reasons given in the text the participation of magnesium, as an ash constituent in tooth development, is different from that of calcium and phosphorus. The ash of the lower incisor has a higher percentage of calcium, a lower percentage of magnesium, and shows no consistent difference in phosphorus from that of the upper tooth.

Puberty conditions an alteration in the orderly progression of the chemical differentiation of the teeth during growth.

I take this occasion to express my deep appreciation of the kindly guidance exercised by Dr. F. S. Hammett in this work and the assistance given me by Mr. H. L. Hinski of the Laboratory of Biochemistry of The Wistar Institute.

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XUM

PHENOL TESTS.*

II. NITROUS ACID TESTS. THE MILLON AND SIMILAR TESTS. SPECTROPHOTOMETRIC INVESTIGATIONS.

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INTRODUCTION.

A general classification of phenol tests, with a bibliography, was given in the first paper of this series (Gibbs, 1926). This article treats of a number of phenol reagents, consisting of nitrous acid, nitrites, alkyl nitrites, dilute nitric acid, and any of the above in presence of salts of mercury. These reagents give red solutions which, in some cases, are delicate, but not characteristic tests for the species. By various modifications and procedures attempts have been made to develop these tests for distinguishing between certain phenols. In some cases fair success has been attained. By comparison of the colors with a known standard, approximate quantitative accuracy has been developed for certain phenols.

For purposes of classification, these tests may be divided into two groups according to the reagents employed.

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1. Nitrous acid and nitric acid reagents.
2. Nitrous acid and nitric acid reagents containing salts of metals.

In the latter are the Millon, Hoffmann, Plugge, Lintner, and similar reagents.

Although Millon's test is the most delicate and the most popular of all of the tests and has been made the subject of numerous researches, I have found in the literature no definite proof of the reactions involved. There will be presented in this paper experimental evidence of the two stage nature of the reactions. The first stage is essentially the formation of nitrosophenols, and subsequent to this is the formation of more highly colored products. This process is catalyzed by the mercury salts and Vaubel (1900) describes a mercury derivative which he isolated and analyzed.

The difference in the behavior of these reagents with phenols, and the discrepancies concerning the delicacy of the tests as performed by different investigators and recorded in the literature, are evidently due to the different proportions of nitric acid, nitrous acid, and mercury salts. These differences affect the speed of the reaction and secondary condensations, and probably do not occasion any fundamental difference in the primary reaction. The most delicate test is obtained when these three ingredients are present in the proper proportion, a condition which seems to be fulfilled best by the freshly prepared Millon reagent. Even in the case of this reagent the method of preparation is an important factor.

When the Millon reagent reacts with phenol, *p*-nitrosophenol is formed, together with a compound which is quite red in acid solution, a distinction from *p*-nitrosophenol. The nature of this compound has not been investigated by me but the evidence points to its being a derivative of *p*-nitrosophenol (see Vaubel, 1900).

This view seems reasonable when it is remembered that the nitrosophenols are quite reactive, unstable in the air, capable of many condensations, and that evidence supports both the nitroso- and the quinone oxime formulas. These desmotropic forms are in equilibrium with each other, act as acid-base indicators, and give colors to the solutions. Moreover, I have found that dilute solutions of *p*-nitrosophenol react with solutions of

mercurous nitrate forming red or brown precipitates which I have not investigated. (See also the work of Ingold and Piggott (1924) on the effect of temperature on the equilibrium $2N{:}O \rightleftharpoons \begin{matrix} -N- & O \\ | & | \\ O- & N- \end{matrix}$ in their studies of nitrosomesitylene in benzene solution).

Of the many colored nitroso compounds (quinone oximes) six commercial products are described by Rowe (1924) in the Colour Index and all are made by the action of nitrous acid on (1) resorcinol, (2) β-naphthol, (3) α-naphthol, (4) 2,7-dihydroxynaphthalene, (5) β-naphthol-6-sulfonic acid, and (6) α-naphthol-4-sulfonic acid. It is evident that the nitrous acid may react in a position ortho to the hydroxyl group and that an unsubstituted position para to the hydroxyl is not always necessary. I have indicated this in an incomplete study of the action of nitrous acid upon a very pure sample of *p*-cresol. Moreover, more than one equivalent of nitrous acid may react, as in the case of resorcinol.

In regard to the effect of the mercury in those tests employing compounds of this metal in connection with nitrous acid, it is only necessary to note the great reactivity of this element with organic compounds, the host of mercury organic derivatives, and the many cases where mercury is employed as a catalyst in organic synthesis, to appreciate the complications that may be introduced by this element as employed in the Millon, Hoffmann, Plugge, Lintner, and other reagents upon various phenols. The effect of mercury is not limited to the formation of mercury organic compounds but may be directional in governing the course of a reaction in substitution. In the presence of condensing agents complicated products well may result (see Whitmore, 1921).

It is interesting to recall that nitrosobenzene was first prepared by the action of nitrosyl bromide upon mercury diphenyl, (von Baeyer, 1874), and it cannot be prepared by the action of nitrous acid on benzene; that *p*-nitrosophenol reacts with nitrous acid and with hydroxylamine to form *p*-diazophenol, $O{:}C_6H_4{:}N_2$, which is susceptible to various couplings forming dyes; and that nitrosobenzenes are capable of polymerization like aldol.

LITERATURE.

A brief review of the most important literature bearing on these tests follows.

1. Nitrous Acid and Nitric Acid Reagents.—Griessmayer (1871) employed potassium nitrite and sulfuric acid in solution and nitrous acid in nitric as reagents. The latter gives a blood-red color with ellagic acid which is stated to be characteristic. (See also Perkin and Nierenstein (1905).) Eykman (1882) described an ethyl nitrite test for phenols claiming a delicacy for phenol of 1 part in 2,000,000. The solution of the phenol was treated with a few drops of an alcoholic solution of ethyl nitrite and then mixed with an equal volume of sulfuric acid, thus producing the red color. Huerre (1922) obtained color reactions with diluted nitric acid and various phenols. Some phenols gave no color, and certain phenol ethers formed nitro derivatives which crystallized out of the solutions. Ware (1925) made use of a dilute solution of nitrous acid and of nitric acid for the detection of certain plant phenols. Ekkert (1925) developed tests for nitrous and nitric acids. He employed a strong hydrochloric acid solution of resorcinol as the reagent and could detect 0.01 mg. of sodium nitrite and 0.05 mg. of potassium nitrate by the color reaction.

2. Nitrous and Nitric Acid Reagents Containing Salts of Metals.—The well known reagents containing mercury are those devised by Millon, Hoffmann, Plugge, and Lintner. Millon (1849, 1850) proposed a solution of mercury in nitric acid as a protein reagent. It has acquired great vogue, especially in the detection of phenolic compounds. Hoffmann (1853) put forth a reagent for tyrosine consisting of a nitric acid solution of mercuric oxide. Meyer (1864) showed that there must also be present a trace of nitrous acid, a condition present in Hoffmann's work but not recognized by him. Plugge (1872) found that a reagent consisting of a solution of mercurous nitrate which contained traces of nitrous acid would detect 1 part of phenol in 200,000. The color with phenol and some other phenols is developed on boiling the solution. Almén (1877) compared various tests for delicacy and found the Millon reaction with phenol most delicate, 1 part in 2,000,000.

In the mercury-nitrous acid class of reagents Hoffmann's test was found to be next in delicacy and Plugge's test least delicate. The great variation in the delicacy of tests with different phenols is brought out in this early work. For example the ferric chloride test will show 1 part of phenol in 3000, and 1 part of salicylic acid in 100,000 while the Millon test has about the same delicacy of 1 part per million for both phenols.

Nasse (1879) believed the action of Millon's reagent to be a nitration, but 20 years later (1901), in light of further information, he changed his view. Hirschsohn (1881) found the delicacy of the Millon test to be 1 part of thymol in 16,000 parts. He also employed among other tests the nitric acid reaction for thymol and phenol. With thymol, 1 part in 32,000,

a pale yellow color is obtained. Nickel (1890) experimented with other salts of mercury, such as mercuric chloride, in the nitrous acid reagent, and he also records some experiments with zinc chloride and zinc sulfate. A number of substances, principally glucosides, were found to give blue or violet color reactions with a reagent consisting of potassium nitrite and zinc sulfate. In commenting on Nasse's view that Millon's reagent produced a nitration, Nickel gave it as his *opinion* that nitrosophenols were formed which then further transformed into red dyes.

Lintner (1900), in his work upon mercuric salicylate, employed the Millon test in a modified form. He states that the action of Millon's reagent is due to mercuric nitrate, nitric and nitrous acids, and that mercurous nitrate plays no rôle in the reaction. The best test for salicylic acid is obtained by first producing the mercuric salicylate by boiling the salicylic acid solution with a few drops of a 10 per cent solution of acid mercuric nitrate, then adding 2 or 3 drops of dilute sulfuric acid and finally sodium nitrite solution, dropwise. The intense red color appears immediately. He believed this to be due to a nitroso derivative. The limit of delicacy is about 1 part of salicylic acid in 500,000. Tests for proteins may be made in the same way.

Vaubel (1900), without definite proof of the first stage of the reaction, describes the action of the Millon reagent as occurring in two stages:

(a) $$C_6H_5OH + HNO_2 = C_6H_4\begin{matrix}NO\\OH\end{matrix} + H_2O$$

(b) $$C_6H_4\begin{matrix}NO\\OH\end{matrix} + 4HgNO_3 + 3NO + 3C_6H_5OH =$$

$$HON = \left(\begin{matrix}-N-OC_6H_4O\\ |\quad\quad\quad |\\ O\text{-}\text{-}\text{-}\text{-}\text{-}\text{-}\text{-}Hg\end{matrix}\right)_4 + 3NO_2 + 3H_2O$$

On heating this mercury derivative with sodium hydroxide solution, it loses its mercury and a compound, apparently of the formula

$$\left.\begin{matrix}\\ NaON\\ \\ \end{matrix}\right\} = (O\cdot C_6H_4\cdot ONa)_2 = (C_6H_4\cdot ONa)_2$$

is formed. This compound is soluble in water and the free acid is precipitated on acidification. He also states that the Millon test is not given by diortho and dimeta substituted compounds.

Nasse (1901) states that the Millon reagent should contain no nitric acid

and that it is best prepared by mixing mercuric acetate solution with a few drops of a 1 per cent solution of an alkali nitrite, and employing the same either with or without the addition of acetic acid. It seems to the writer that some nitric acid will be present under these conditions.

Bach (1911) employed the Millon test for phenols in waste waters and found the delicacy to be less than 1 part phenol in 200,000 parts. Elvove (1917) pointed out that the delicacy of the Millon test depends upon the mode of preparing the reagent and the manner of its use, and gives details for the preparation of an efficient reagent. By using comparatively large amounts of the reagent it is possible to detect and to estimate quantitatively phenol and the cresols without the usual procedure of heating the reaction mixture. He found the delicacy of the test to be 1 part of phenol in 600,000, and he also observed that a sample of cresol containing some phenol could be differentiated from a sample of pure cresol by its behavior with the Millon reagent. Chapin (1920) noted the qualitative observations of Elvove and developed a quantitative method for determining phenol in the presence of the three cresols. He claimed a fair accuracy for the method. He also presented a mathematical analysis of his colorimetric procedure and tabulated the colors obtained with a number of phenols, both mono- and polyhydric.

EXPERIMENTAL.

In order to investigate the hypothesis that the primary reaction between phenols and the nitrous acid and nitric acid reagents results in the formation of a nitrosophenol, a study of the development of color at room temperature was made in the following solutions.

Phenol.

Mixture 1.—50 cc. of phenol solution, 2:1000 in water, were treated with 10 cc. of water containing 10 drops of concentrated nitric acid of the best laboratory reagent quality.

Mixture 2.—Mixture 1 plus 1 cc. of a concentrated solution of mercurous nitrate to which just sufficient nitric acid had been added to prevent turbidity.

Mixture 3.—50 cc. of phenol solution, 2:1000 in water, were treated with 10 cc. of water containing 2 drops of a concentrated solution of sodium nitrite and 1 drop of concentrated hydrochloric acid.

Mixture 4.—Mixture 3 to which was added 1 cc. of the concentrated solution of mercurous nitrate as in No. 2.

Mixture 5.—50 cc. of phenol solution, 2:1000, plus 10 cc. of Millon's reagent.

Mixture 6.—50 cc. of phenol solution, 2:1000, plus 10 drops of Millon's reagent.

The Millon reagent employed in these tests was prepared according to Elvove (1917) as follows: 50 cc. of concentrated nitric acid, density 1.405 at 25°, were added to 68 gm. of mercury in a 250 cc. beaker. The resulting solution, measuring about 40 cc., was diluted with 92 cc. of water. To this were added 2.76 cc. of nitric acid and mixed until the precipitate, which often formed, was completely dissolved.

The red color developed most rapidly in Mixture 5. The color development in Mixtures 2, 4, and 6, all containing mercury, was quite rapid and satisfactory to about the same degree in a given time. By adjusting the proportions of mercury compound used, the color development may be made approximately equal in the various mixtures. A crystal of mercurous nitrate dropped into the nitric or nitrous acid tests accelerates the formation of the red color in the same way as the solution of mercurous nitrate. Some experiments employing mercuric chloride in place of the nitrite indicate that this compound of mercury possesses the catalytic power to a much lesser degree.

The production of color in Mixtures 1 and 3 was at first a yellow shade and was most rapid in No. 3, the nitrite solution. By increasing the concentration of the nitric acid in Mixture 1 the color development was greatly accelerated. On long standing the colors in Mixtures 1 and 3 became red but not quite the same shade as the solutions containing mercury.

On precipitating the mercury from any of the solutions by the addition of ammonia or sodium hydroxide and filtering there was obtained a clear red solution. By adjustment of the concentration and the pH value of these solutions they can be brought to match the color of the solutions obtained by the nitric acid test, No. 1, and the nitrous acid test, No. 3, when the latter solutions are similarly adjusted for concentration and pH. Neither of these tests, Nos. 1 and 3, employed mercury compounds. The pH of the various mixtures was adjusted by diluting a measured volume with buffers of the Clark and Lubs series (Clark, 1925). At the higher pH ranges the colors were reddish brown, and in the acid ranges yellow.

The test for nitrosophenol was performed on all the mixtures

as follows: In the case of Mixtures 1 and 3, the addition of concentrated sulfuric acid produced the condensation of nitrosophenol and phenol to indophenol, the Liebermann reaction.

In the case of the solutions containing mercury (Mixtures 2, 4, 5, and 6) the result was obtained on the red solutions after precipitating the mercury with sodium hydroxide. In some cases it was found that the test could not be obtained until after a little phenol solution, 2:1000, had been added to the red solution to be tested. This is evidently due to the fact that all of the phenol originally present had reacted with the nitrous acid reagent.

In order to obtain some idea of the effect of pH on the rate of the reaction between phenol and nitrous acid, 5 cc. portions of phenol solution (2:1000 = 0.0213 M) were mixed with equal quantities of sodium nitrite solution (0.0213 M) and to this were added 10 cc. of buffers pH 1.2, 2, 3, 4, 5, 6, 7, 8, 9, and 10. These mixtures were allowed to stand 2 days at room temperature. The color gradually developed in the acid ranges, first yellow then slowly deepening to red, no color showing at pH 7, 8, 9, and 10. The best color development seems to be in the range pH 4 to 5. On heating the solutions the rate of color formation was very much accelerated.

The production of the characteristic color test with phenol and dilute nitric acid was proven to be due to nitrous acid, which was either present in the nitric acid or developed by the reducing action of the phenol, by the following tests.

Solutions of three different concentrations of phenol in water were treated with dilute nitric acid and to identical comparison solutions there were added small quantities of urea for the purpose of destroying any nitrous acid that might be present.

In every case the color development was normal in a few hours except where the urea had been added. In the latter experiments no color developed and some gas was evolved from the solutions. On making the test solutions alkaline at the end of the experiment, no color was developed in the solutions containing urea, while the color in the solutions containing no urea was greatly enhanced.

In order to compare with *p*-nitrosophenol the properties of the colored compounds obtained in the tests described above, a sample of *p*-nitrosophenol was prepared by the action of nitrous acid on phenol and purified as follows. The product was put into

hot alcohol containing decolorizing carbon, shaken, and quickly filtered into cold water containing a very little acetic or hydrochloric acid. On cooling with ice there was obtained a good crop of crystals. After repeating the process several times, a beautiful crop of almost colorless crystals was obtained. They were rapidly separated by filtration with as little exposure to the air as possible. Without further drying, a portion of these crystals was dissolved in warm water, which had been previously boiled to expel air, the solution cooled to room temperature, and filtered. Analysis for nitrogen gave the following results.[1]

Nitrogen by Kjeldahl.

I. 25 cc. solution = 0.00658 gm. N.
II. 25 " " = 0.00658 " "

Calculated, *p*-nitrosophenol equals a concentration of 2.316 gm. per 1000 cc. or 0.0188 molar.

The dissociation constant of this preparation of *p*-nitrosophenol, determined by the method of Salm (1906) is 6.6 in terms of pK_a.

The solutions employed were: for the acid solution 0.5 cc. of *p*-nitrosophenol solution plus 10 cc. of buffer pH 3; for the alkaline solution 0.5 cc. of *p*-nitrosophenol solution plus 10 cc. of 0.2 N sodium hydroxide. Superpositions of these were matched against 1 cc. of solution plus 9.5 cc. of buffer pH 6.6 and 10 cc. of water.

The dissociation constant was also determined by the spectrophotometric method (Holmes, 1924). Two 2 cc. portions of a solution of pure *p*-nitrosophenol were diluted with 20 cc. of 0.2 N sodium hydroxide and with 20 cc. of buffer pH 2, and the transmittancy measured in 10 cm. tubes at wave-length 560 $m\mu$ as described under the heading "Absorption spectra."

Solution.	T	− log T
0.2 N sodium hydroxide	0.1075	0.968
pH 2	1.00	0.0

Since the solution in 0.2 N alkali gave the maximum color obtained in alkaline solutions, it is assumed that all of the compound is here present in the colored form and in the solution at pH 2 the colored form is entirely absent. Therefore at the value 0.968 ÷ 2 = 0.484, the two forms are present in equal quantities.

[1] I have Mr. C. G. Remsburg of this laboratory to thank for the analyses.

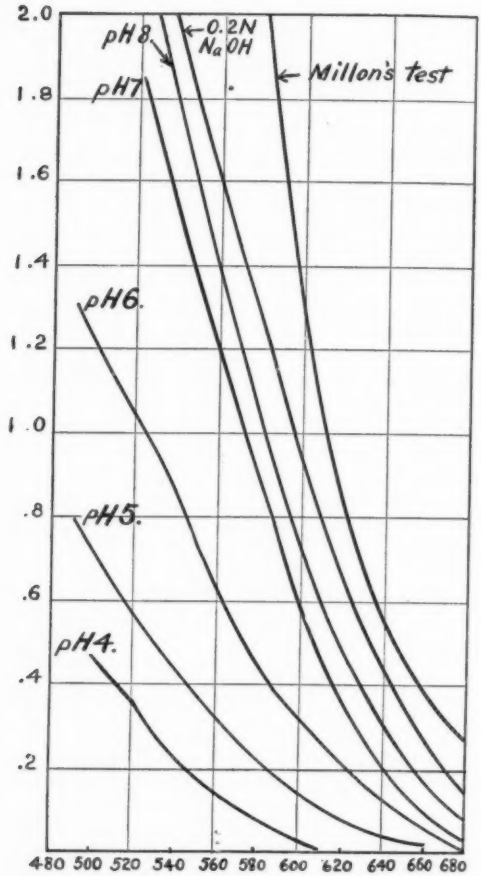

FIG. 1. Spectrophotometric absorption curves of *p*-nitrosophenol at pH 4, 5, 6, 7, 8 and 0.2 N sodium hydroxide at concentration of 0.0019 M, and the color produced by Millon's reagent acting on phenol. Ordinates, −log transmittancy; abscissæ, wave-lengths in $m\mu$.

A solution of 2 cc. of the *p*-nitrosophenol in 20 cc. of buffer pH 6.4 gave the value T = 0.33, −log T = 0.482. This value of 6.4 for pK_a is less than that obtained by the method of Salm and I

believe it to be more accurate, because of the difficulty of matching color when Salm's method is applied to such weakly colored solutions. It is possible that the rather rapid fading of the solution in 0.2 N sodium hydroxide may have reduced the pK_a value somewhat.

A comparison of the above value was made with that of the color obtained in the Millon test as follows: 50 cc. of phenol solution, 2:1000, were treated with 25 cc. of Millon's reagent. A brilliant deep red color developed in about 0.5 hour. This solution was treated with a 10 per cent solution of sodium hydroxide until no more precipitate formed. It was then filtered, and the red filtrate approximately neutralized with 0.1 N sulfuric acid solution. This clear red-brown solution showed substantially the same dissociation value, pK_a 6.6, as that found in the case of the solution of pure *p*-nitrosophenol and also the same spectrophotometric absorption curve when adjusted for concentration and pH values (see Fig. 1).

Although *p*-nitrosophenol is present and probably the first compound formed, it is not the only colored derivative produced by the action of Millon's reagent as shown by the following investigation of the mercury-sodium hydroxide precipitate from the above experiment.

This precipitate was washed with water until the filtrate appeared practically colorless, and then it was treated on the filter with dilute nitric acid. The filtrate appeared deep red and did not lose color on further acidification with nitric acid. The absorption spectrum of this *acid* solution in the visible region of the spectrum, very closely approximates the curves for *p*-nitrosophenol in the *alkaline* ranges. The curve has the same slope, shows no tendency to develop bands in this region of the spectrum, and the intense absorption of the acid solution is quite a distinction from *p*-nitrosophenol.

No further investigation of this color was attempted to determine its constitution or how it is carried down with the precipitation of the mercury by sodium hydroxide.

Absorption Spectra.

The absorption spectra of the pure solution of *p*-nitrosophenol, previously described, were measured in the visible region at

The reaction with phenol produces *p*-nitrosophenol which may condense to produce other color compounds. The presence of mercury compounds accelerates the color formation.

The pK_a value of pure *p*-nitrosophenol was determined by the method of Salm to be 6.6 and by the spectrophotometric method 6.4. The latter is believed to be more accurate. The dissociation constant for a color compound formed by the Millon reaction was found to be 6.6.

The absorption curves in the visible region are recorded for *p*-nitrosophenol at pH 4, 5, 6, 7, 8 and 0.2 N sodium hydroxide and for the color produced by the Millon reagent.

Para-cresol and tyrosine react with Millon's reagent and with nitrous acid. The substitution of groups in the para position to the hydroxyl in general does not seem to interfere with the nitrous acid tests.

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Ware, A. H., The detection of plant phenols by the use of nitrites or nitric acid, *Analyst*, 1925, l, 384.

Whitmore, F. C., Organic compounds of mercury, New York, 1921.

XUM

various pH levels by means of a Keuffel and Esser color analyzer. 2 cc. quantities of the solution were diluted with 20 cc. quantities of buffers pH 4, 5, 6, 7, 8 and 0.2 N sodium hydroxide, making solutions 0.0019 molar, which were placed in 10 cm. tubes. The curves are plotted in Fig. 1.

There is also plotted on this chart the absorption curve of a phenol solution treated with a few drops of Millon's reagent. Owing to the rapidity of the color development the curve is difficult to obtain accurately. The readings were commenced at 700 $m\mu$ and proceeded toward the short wave-lengths and since the absorption in the blue was increasing rapidly the curve is too steep at the upper end. Disregarding this discrepancy the conformity with the *p*-nitrosophenol type of curve is satisfactory.

Para-Cresol.

The reactivity of phenols that have the position para to the hydroxyl group unsubstituted is well recognized and in these cases the formation of various derivatives through reactions with the para-hydrogen is easily understood. In cases where the para position is substituted, especially by a methyl group as in the case of para-cresol, certain types of reactions may proceed with difficulty or not at all (compare Pummerer *et al.*, 1922, 1925, mentioned under ferricyanide reagents in Paper I). Since the Millon color test is in general so delicate and might be attributed to impurities of ortho- or meta-cresol contaminating para-cresol, it was decided to prepare this cresol in a pure state to determine its reactivity not only with Millon's reagent but also with nitrous acid and some other compounds.

The purification by a new procedure will be described in a later paper.

Millon Test.—Para-cresol gives the Millon test in concentration of 1:1000 very deep red and 1:100,000 pink.

Nitrous Acid Test.—At concentrations of 1:2000 *p*-cresol gives a positive coloration with nitrous acid. In performing the test equal volumes of solutions of *p*-cresol 1:1000 and sodium nitrite of the same molar concentration were mixed and acidified with hydrochloric acid.

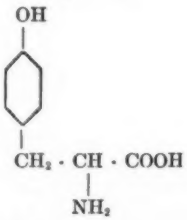
In order to establish further the reactivity of *p*-cresol with nitrous acid 0.1 mol (10.8 gm.) was put into water, 0.1 mol

sodium hydroxide (4.0 gm.) dissolved in water, added and then 0.1 mol sodium nitrite (7.0 gm.) dissolved in water was added. The total volume was 250 cc.

This solution was surrounded by an ice bath, chilled to between 0° and 7°, and a solution of 25 gm. of sulfuric acid in 100 cc. of water slowly dropped in while the solution was thoroughly agitated.

At the low temperatures there was very little evidence of a reaction between the *p*-cresol and nitrous acid, the former separating as the solution becomes acid. After all of the sulfuric acid had been added the solution was slowly warmed to room temperature and then there was evidence of a reaction through the formation of a red precipitate. On distilling with steam very little of the *p*-cresol was recovered, the solution becoming red and much resinous material forming. The products of the reaction were not investigated since the study seemed hardly pertinent to this work.

Tyrosine.



Dilute solutions of tyrosine, at room temperature, treated with dilute nitric or nitrous acid, slowly develop a color first yellow and gradually deepening. The addition of mercurous nitrate to the solutions accelerates the color formation. Millon's reagent develops the red color in the same way. The color formation in the case of tyrosine is much slower than with phenol and the behavior of the color solutions is similar in changing the pH. No study has been made of the colored compound formed but it is evidently due to the action of nitrous acid.

SUMMARY.

The phenol reagents, nitrous acid, dilute nitric acid, and Millon's, have been studied, and the literature reviewed.

The reaction with phenol produces *p*-nitrosophenol which may condense to produce other color compounds. The presence of mercury compounds accelerates the color formation.

The pK_a value of pure *p*-nitrosophenol was determined by the method of Salm to be 6.6 and by the spectrophotometric method 6.4. The latter is believed to be more accurate. The dissociation constant for a color compound formed by the Millon reaction was found to be 6.6.

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NOTE ON THE ACTION OF AMMONIA ON PROPYLENE OXIDE.

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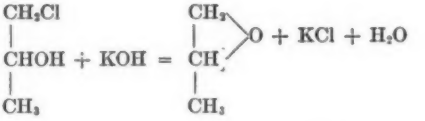
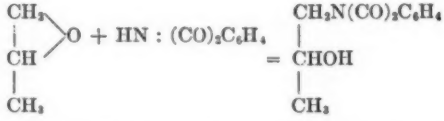
(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 1, 1926.)

In a previous communication on the configurational relationships of lactic and 3-hydroxybutyric acids,[1] a method was given for the preparation of aminohydroxypropane by the action of ammonia on propylene oxide. It is generally assumed that under such conditions the amino group enters position (1) and the hydroxyl position (2). The reason for the assumption is the following: It had been found that 1-amino-2-hydroxypropane was formed by the action of potassium phthalimide on either 1-chloro-2-hydroxypropane or on 1-hydroxy-2-chloropropane. This result can be explained on the assumption that the first step of the reaction consists of the formation of propylene oxide which is subsequently ammonolized to form 1-amino-2-hydroxypropane.[2]

The set of reactions leading to the formation of the base, then, may be expressed as follows:





[1] Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxviii, 415.

[2] Gabriel, S., *Ber. chem. Ges.*, 1916, xlix, 2120. Gabriel, S., and Ohle, H., *Ber. chem. Ges.*, 1917, l, 804.

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Thus, *a priori*, it is to be expected that the amine prepared by the action of ammonia directly on the oxide also has the composition of 1-amino-2-hydroxypropane. This amine and its isomer can be easily differentiated from each other by the melting points of the two isomeric hydroxypropylphenylthioureas derived from them. The derivative of 1-amino-2-hydroxypropane has a melting point of 108–109°C., whereas that of the isomer is at 141°C. The substance prepared by us melts at 107–108°C., thus showing the correctness of the assumption that on addition of ammonia to propylene oxide 1-amino-2-hydroxypropane is formed.

Incidentally it may be added that under the conditions adopted by us the formation of secondary and tertiary bases is reduced to a minimum.

EXPERIMENTAL.

Propylene Oxide.—A double neck distilling flask containing 65 gm. of potassium hydroxide dissolved in 85 cc. of water was immersed in a water bath. The flask, which held a dropping funnel containing 95 gm. of propylene chlorohydrin, was connected with a condenser through which water at 0°C. was circulated. The temperature of the water bath was then raised to 50°C. When this temperature was reached, one-third of the chlorohydrin was added to the alkaline solution and the temperature was quickly raised to 60°C. When the condensation of the propylene oxide became slow another one-third of the chlorohydrin was added, and finally the rest was added. The flask was vigorously shaken to drive over the propylene oxide. 49 gm., or 84 per cent of the theoretical yield, of propylene oxide were thus obtained.

Aminoisopropyl Alcohol.—15 gm. of the propylene oxide described above were poured into 2 liters of concentrated aqueous ammonia at 0°C. and allowed to stand in a refrigerator overnight. The solution was then concentrated under reduced pressure at room temperature until the odor of ammonia disappeared. The residue, about 25 cc., was taken up in 60 cc. of absolute alcohol. This solution was cooled to 0°C. and anhydrous potassium carbonate was added and well mixed. The dry alcoholic solution was decanted and the remaining potassium carbonate paste was extracted several times with absolute alcohol, adding fresh portions of potassium carbonate each time. The combined alcoholic

extracts stood for a few hours over anhydrous potassium carbonate. The alcohol was then removed under reduced pressure and the aminoisopropyl alcohol distilled at 156–162°C. at 761 mm. The yield was about 70 per cent. It analyzed as follows:

0.0834 gm. substance: (Van Slyke) 5.67 cc. N_2 at 25°C. and 764.9 mm.

C_3H_9ON.	Calculated.	N 18.66.
	Found.	" 18.96.

In the distilling flask there remained a little of a higher boiling residue which crystallized on standing and apparently represented di-(hydroxypropyl) amine, or tri-(hydroxypropyl) amine, or a mixture of both.

If the extraction with absolute alcohol and anhydrous potassium carbonate is omitted and the residue, after the ammonia has been removed by distillation under diminished pressure, is directly distilled at ordinary pressure, the temperature rises constantly from 100–160°C. or above, so that only a very small amount of the amine boiling correctly can be obtained.

2-Hydroxypropylphenylthiourea. $CH_3CHOHCH_2NHC(=S)NHC_6H_5$

To 2 gm. of aminoisopropyl alcohol were added 3.7 gm. of phenylisothiocyanate, the addition being accompanied by the evolution of heat. The substance thus obtained was recrystallized four times from water. It melted at 107–108°C. and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 9.48 cc. 0.1 N HCl.

$C_{10}H_{14}ON_2S$.	Calculated.	N 13.33.
	Found.	" 13.12.

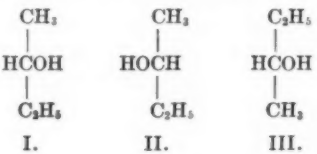
THE CONFIGURATIONAL RELATIONSHIP OF DEXTRO-METHYLETHYL CARBINOL TO DEXTRO-LACTIC ACID.

By P. A. LEVENE, A. WALTI, AND H. L. HALLER.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 1, 1926.)

The configurations of aliphatic substances containing in their molecules several secondary alcoholic groups, or one or more secondary alcoholic groups in addition to a polar radicle as $-COOH$, $-CHO$, or $=C=O$, have been correlated to a single reference substance, lactic acid. Nothing is known, however, regarding the stereochemical relationships of simple secondary alcohols to the same substance of reference, nor is there any definite knowledge as to the stereochemical relationships of individual secondary alcohols among themselves. The case of secondary alcohols is complicated by the following fact. The first member of the series is methylethyl carbinol (I). Its

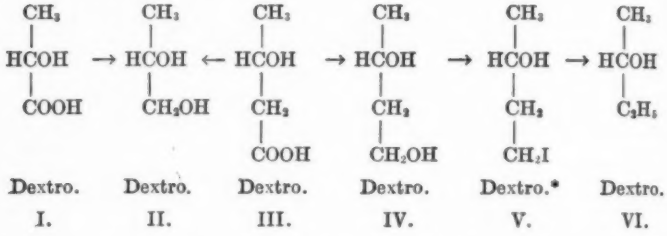


I. II. III.

enantiomorph (II) may be represented also as ethylmethyl carbinol (III). A question has been raised as to whether or not the higher homologues of the methyl and ethyl series should rotate in opposite directions as their first members do. Two conflicting answers have been given to the question, both based on indirect evidence.[1]

[1] Clough, G. W., *Proc. Chem. Soc.*, 1913, xxix, 357. Kenyon, J., *J. Chem. Soc.*, 1914, cv, 2226.

In the present communication data are given which permit the correlation of the configuration of dextro-methylethyl carbinol with that of dextro-lactic acid. The arguments are presented in the following figures:



The reactions leading to the correlations of (I) to (IV) were described in previous communications by Levene and Haller[2] and Levene and Walti.[3] The remaining reactions are described below. Thus dextro-methylethyl carbinol is correlated to dextro-lactic acid, which belongs to the *l* series; hence, dextro-ethyl-methyl carbinol also belongs to the *l* series.

EXPERIMENTAL.

Preparation of Dextro-1, 3-Dihydroxybutane.—Dextro-1, 3-dihydroxybutane was obtained by reducing freshly prepared aldol with brewer's yeast according to the procedure of Neuberg and Kerb.[4] It has been found unnecessary to distil the aldol after removal of the ether under reduced pressure. The glycol showed a rotation of $\alpha = +9.9°$ in a 1 dm. tube.

The glycol obtained by the use of brewer's yeast had a specific rotation higher than that of the product obtained when baker's yeast was employed. A glycol obtained with baker's yeast gave $\alpha_D^{20} = +1.6°$ to $+2.5°$ in a 1 dm. tube, whereas the glycol obtained with brewer's yeast gave $\alpha_D^{20} = +9.9°$ in a 1 dm. tube.

* The rotation of the iodohydrin could not be measured because of the dark color of the solution. The bromo derivative, however, was dextrorotatory.

[2] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, 1925, lxv, 49; 1926, lxvii, 329; 1926, lxix, 165.

[3] Levene, P. A., and Walti, A., *J. Biol. Chem.*, 1926, lxviii, 415.

[4] Neuberg, C., and Kerb, E., *Biochem. Z.*, 1918, xcii, 96.

Diurethane of Dextro-1, 3-Dihydroxybutane.—To 0.9 gm. of 1,3-dihydroxybutane were added 2.5 gm. of phenylisocyanate and the mixture was heated at 100° for 1 hour. The solid product was washed with petroleum ether, and was recrystallized three times from dilute alcohol. The diphenylurethane melted at 127–129°C. and analyzed as follows:

0.0500 gm. substance: (Kjeldahl) 3.10 cc. 0.1 N HCl.

$C_{18}H_{20}O_4N_2$.	Calculated.	N 8.53.
	Found.	" 8.68.

It had the following rotation in absolute alcohol.

$$[\alpha]_D^{20} = \frac{+3.45° \times 100}{1 \times 6.48} = +53.24°.$$

Preparation of 1-Iodo-3-Hydroxybutane.—Into 40 gm. of dextro-1, 3-dihydroxybutane 57 gm. of dry hydrogen iodide (1 equivalent) were passed, the temperature being maintained at 0°C. The dark solution was heated in a water bath for ½ hour. After cooling, 100 cc. of chloroform, 10 gm. of ice, and 5 cc. of water were added and the mixture was made neutral to Congo red with sodium carbonate. The chloroform was separated and the extraction with chloroform was repeated twice. The combined chloroform extracts were dried over sodium sulfate. The chloroform was removed by distillation under reduced pressure and the 1-iodo-3-hydroxybutane was distilled. It boiled at 58–60°C., p = 0.4 to 0.5 mm. The yield was 39 gm. The substance analyzed as follows:

0.1031 gm. substance: 0.1236 gm. AgI.

C_4H_9OI.	Calculated.	I 63.50.
	Found.	" 64.79.

Owing to the dark color of the solution it was not possible to measure its optical rotation.

Preparation of Dextro-1-Bromo-3-Hydroxybutane.—This product was obtained by passing 1 molecule of dry hydrogen bromide into 1 molecule of dextro-1, 3-dihydroxybutane ($\alpha = +9.9°$) at 0°C. The acid mixture was neutralized and worked up in the same way as the above mentioned 1-iodo-3-hydroxybutane. The dextro-1-bromo-3-hydroxybutane boiled at 72–76°C. at 20 mm. and had a rotation of $\alpha_D^{20} = +2.53°$ in a 1 dm. tube.

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It analyzed as follows:

0.1413 gm. substance: 0.1740 gm. AgBr.

C_4H_9OBr.	Calculated.	Br 52.3.
	Found.	" 52.4.

Reduction of 1-Iodo-3-Hydroxybutane to Dextro-Methylethyl Carbinol.—60 cc. of a 10 per cent aqueous sodium hydroxide solution and 19 gm. of 1-iodo-3-hydroxybutane were placed in a shaking flask and the flask was then connected with a hydrogen cylinder. After replacing the air in the flask by hydrogen, 0.1 gm. of colloidal palladium in a little water was added. The absorption of hydrogen was rapid at first. The reduction was continued for 48 hours. Two 0.1 gm. portions of colloidal palladium were added during the course of the reaction. The reaction mixture was extracted with ether in a continuous extraction apparatus. The ether extract was carefully dried with sodium sulfate and then with 0.1 gm. of metallic sodium. The ether was distilled off, employing a fractionating column. From the residue of two such experiments the following fractions were obtained by fractional distillation.

95– 99°C.	= 1.5 cc.	
99–104 "	= 3.5 "	$\alpha_D^{20} = +3.54°$ in 1 dm. tube.
104–195 "	= 2.5 "	$\alpha_D^{20} = +2.69$ " 1 " "

The residue from the distillation was about 0.3 cc.

Phenylurethane of Methylethyl Carbinol.—To 0.74 gm. of the fraction boiling from 99–104°, 1.4 gm. of phenylisocyanate were added. The mixture was heated at 100–120° for 4 hours. Crystallization took place on standing overnight The product was recrystallized three times from dilute alcohol. It melted at 62°C. The rotation of the substance in absolute alcohol was

$$[\alpha]_D^{22} = \frac{+0.81° \times 100}{1 \times 10} = +8.1°.$$

The substance analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.40 cc. 0.1 N HCl.

$C_{11}H_{15}O_2N$.	Calculated.	N 7.25.
	Found.	" 7.56.

The above results were further confirmed by converting levo-methylethyl carbinol into its phenylurethane. The levo-methyl-

ethyl carbinol was obtained by resolution of the inactive alcohol following the procedure of Pickard and Kenyon.[5]

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{23} = \frac{-1.12° \times 100}{1 \times 12.85} = -8.71°.$$

The phenylurethane was prepared in the usual way. Several recrystallizations from 75 per cent alcohol gave a product which melted at 64–65°C. and analyzed as follows:

0.1000 gm. substance: (Kjeldahl) 5.26 cc. 0.1 N HCl.

$C_{11}H_{15}O_2N$.	Calculated.	N 7.25.
	Found.	" 7.28.

In absolute alcohol it had the following rotation.

$$[\alpha]_D^{23} = \frac{-0.88° \times 100}{1 \times 5.25} = -16.76°.$$

[5] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, 1911, xcix, 64.

LACTONE FORMATION OF LACTO- AND MALTOBIONIC ACIDS AND ITS BEARING ON THE STRUCTURE OF LACTOSE AND MALTOSE.

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(Received for publication, November 23. 1926.)

The knowledge of the structure of disaccharides was considerably advanced recently, first by the improvement in the use of methylation methods and in the handling of the methylated sugars, and furthermore by the introduction by Zemplen of the method of gradual degradation.

The method of methylation as well as the method of Zemplen is very laborious and requires considerable quantities of material. Therefore, any simple method yielding information as to the point of union of the two sugars should be found useful.

Recently Levene and Simms[1] have shown that sugar acids in which positions (4) and (5) are not substituted form in aqueous solution two lactones. These differ in the rates of formation and in their concentrations at the points of equilibrium. The six-membered ring is formed rapidly and at the point of equilibrium the lactone has a concentration of 20 to 30 per cent of the original acid; the five-membered ring is formed slowly and at the point of equilibrium the lactone has a concentration of 75 to 80 per cent. Thus it ought to be possible from observations on lactone formation to judge as to the part positions (4) and (5) play in the union between the components of a given disaccharide.

These considerations led us to investigate the lactone formation of two acids, namely lactobionic and maltobionic acids. The lactone formation was followed by observations on the changes in optical rotation and by titration. It must be recalled at this

[1] Levene, P. A., and Simms, H. S., *J. Biol. Chem.*, 1926, lxviii, 737.

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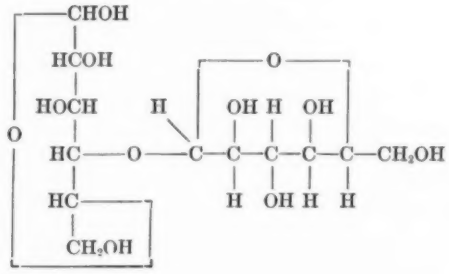
time that the velocity of hydrolysis of the <1,5> lactones is so rapid that it is not possible to detect their presence by titration.

The results of our observations are the following.

Maltobionic acid showed a slight rise in rotation which reached its maximum after 2 hours. The neutralization value remained constant throughout the experiment, which lasted 24 hours, and corresponded accurately to maltobionic acid, namely to 377.

Thus the conclusion is warranted that maltobionic acid forms a six-membered lactone. This conclusion leads to the assumption of a union in position (4) as follows:



This is one of the two possible structures formulated simultaneously by Irvine and Black[2] and by Cooper, Haworth, and Peat.[3]

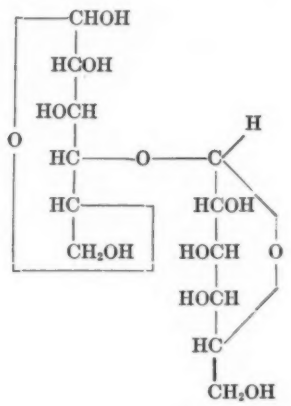
Lactobionic acid in aqueous solution showed a small but definite mutarotation and on titration gave a constant value throughout the experiment, which lasted 24 hours. Again in this case a <1,4> lactone was not formed. This observation was to be expected on the basis of the work of Haworth and Leitch[4] and of Zemplen[5] who assign to lactose the following structure.

[2] Irvine, J. C., and Black, I. M. A., *J. Chem. Soc.*, 1926, cxxix, 862.

[3] Cooper, C. J. A., Haworth, W. N., and Peat, S., *J. Chem. Soc.*, 1926, cxxix, 876.

[4] Haworth, W. N., and Leitch, G. C., *J. Chem. Soc.*, 1918, cxiii, 188.

[5] Zemplen, G., *Ber. chem. Ges.*, 1926, lix, 2402.



EXPERIMENTAL.

Mutarotation of Maltobionic Acid.—The calcium salt of maltobionic acid was used for the experiment. A convenient way of purifying the calcium salt of maltobionic and lactobionic acids is the following. To a 25 per cent solution of the bionic acid were added 1½ volumes of 95 per cent alcohol. The calcium salt settled out as a sticky semifluid mass. The supernatant fluid was decanted, the residue was dissolved in a minimum of water, and the solution was poured gently with stirring into a large excess of methyl alcohol. Two or three reprecipitations sufficed to obtain a calcium salt having the composition required for the salt of a bionic acid.[6]

Maltobionic Acid.—1.0 gm. of the air-dried calcium salt containing 6 per cent moisture was dissolved in 20 cc. of water containing exactly 1 equivalent of hydrochloric acid. 1 cc. of this solution required for neutralization 1.25 cc. of 0.1 N alkali, thus giving 377 as the equivalent weight of the maltobionic acid, which is exactly the value required by theory. The titration values and the changes in rotation are given in Tables I and II.

Lactobionic Acid.—1.0 gm. of the air-dried calcium salt containing 0.8864 gm. of the anhydrous substance was dissolved in

[6] We wish to acknowledge our indebtedness to Dr. Walther F. Goebel for a sample of maltobionic acid.

TABLE I.

Titration of 1 Cc. of a 0.125 N Solution of Maltobionic Acid with 0.1 N Alkali.

Time.	0.1 N alkali.
hrs.	*cc.*
0	1.25
2	1.24
3	1.25
4	1.25
21	1.25
23	1.25
26	1.25
29	1.25
31	1.25

TABLE II.

Changes in Rotation of the Above Solution.

$l = 2$ dm. $t = 24°$C. $\lambda = 5892$ Å.

Time.	α^*
hrs.	
0	9.58
1	10.69
2	10.72
3	10.73
4	10.74
6	10.73
23	10.74

* Accuracy of instrument = ± 0.02°.

TABLE III.

Titration of 1 Cc. of a 0.117 N Solution of Lactobionic Acid with 0.1 N Alkali

Time.	0.1 N alkali.
hrs.	*cc.*
0	1.17
1	1.18
2	1.18
4	1.18
6	1.18
21	1.18
23	1.18
26	1.19
28.5	1.18
30	1.18

TABLE IV.

Changes in Rotation of the Above Solution. Two Experiments Recorded.
$l = 2$ dm. $t = 24°$C. $\lambda = 5892$ Å.

Time.	α^*	
	Experiment I.	Experiment II.
hrs.		
0	1.62	1.66
0.25	1 75	
0.50	1 85	
1	1.96	1 92
2	2.02	2.03
3	2.02	2 03
4		2.06
5	2.03	
6		2.04
7	2 03	
8		2 05
24	2.03	2.06

* Accuracy of instrument = ± 0.02°.

20 cc. of water containing exactly 1 equivalent of hydrochloric acid. 1 cc. of this solution required for neutralization 1.17 cc. of 0.1 N alkali, thus giving for the equivalent weight of the salt the value of 377, which is exactly that required by theory. The solution was then used for titrations and for the observations on changes of optical rotation as a function of time. The results are recorded in Tables III and IV.

THE NUTRITIVE VALUE OF PLASTEIN.

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(Received for publication, November 17, 1926.)

Plastein formation was first observed by Danilevski and Okuneff (1), who showed that if rennin is brought into contact with a solution of proteoses a precipitate is formed. They regarded this precipitate as a resynthesized protein. Since that time several investigators, Kurajeff (2), Nürnberg (3), Grossmann (4), and Sawjalow (5), have studied this so called plastein formation. The last named investigator thought that it is an assimilation product of first importance, and that it is the intermediate product between digested proteins and the formation of blood proteins.

Among the more recent investigators, Henriques and Gjaldbäk (6, 7) also demonstrated that pepsin could induce plastein formation from peptic digests, and they believed it to be a synthetic product, possibly a mixture of proteoses. Levene and Van Slyke (8, 9) carried out a complete hydrolysis of plastein, and found that it yields at least thirteen amino acids. They were also inclined to the view that it is proteose rather than protein in nature.

It has long been a disputed question as to whether plastein is merely a digestion product, a resynthesis of the original protein, or a new synthetic product differing from the protein from which the proteoses are obtained. The recent investigations of Wasteneys and Borsook (10–13) would seem to be conclusive evidence that it is a true synthetic product of protein nature. It thus seemed of interest to test this hypothesis further by feeding tests. The object of this investigation was, therefore, to ascertain whether this unique and evidently "artificial" product exhibits the properties of a so called protein when judged by the feeding method.

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EXPERIMENTAL.

Hydrolysis.—The commercial egg white of Innis Speiden was employed for the preparation of plastein. 1 liter of a 6 per cent solution was brought to a pH of 1.7 electrometrically, and then digested at 37°C. with 3 gm. of Eimer and Amend's pepsin. Chloroform was added as a preservative. The reaction was adjusted to pH 1.7 at frequent intervals during the hydrolysis. At the end of 10 to 15 days no precipitate was observed in the presence of 3 per cent trichloroacetic acid.

Synthesis.—The hydrolysate was next concentrated on the water bath to 40 per cent, calculated on the basis of original protein, brought to a pH 4, 1 per cent pepsin added, and the whole placed in large beakers in an electric oven at 60°C. After 4 hours the plastein was separated by dilution, filtration, washing with water, and drying. The final product was crystalline and resembled the original egg white, except that it was darker in color.

Several attempts were made in the present investigation to determine whether the precipitate would form in the absence of the pepsin, with the result that no formation took place until it was added, thus clearly demonstrating the reversibility of the enzyme action.

Female white mice were used as the experimental animals. The feeding and caging were the same as that described elsewhere (14). The control group received coagulated egg white, while the experimental animals received an equivalent amount of plastein, synthesized from the egg white. The composition of the diets was as follows:

Control.		Per cent of total calories.	Experimental.		Per cent of total calories.
	gm.			gm.	
Coagulated egg white	31	25	Plastein	31	25
Starch	38	31	Starch	38	31
Crisco	21	39	Crisco	21	39
Salt mixture*	7		Salt mixture*	7	
Cod liver oil	3	5	Cod liver oil	3	5
Dried yeast, 100 mg. tablet daily			Dried yeast, 100 mg. tablet daily		

*Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, xxxvii, 572.

Chart 1 shows that growth was somewhat slower with plastein than with egg white. The food intake of the experimental animals was in practically all cases the same as that of the controls. If the curves are examined at the end of the experimental period

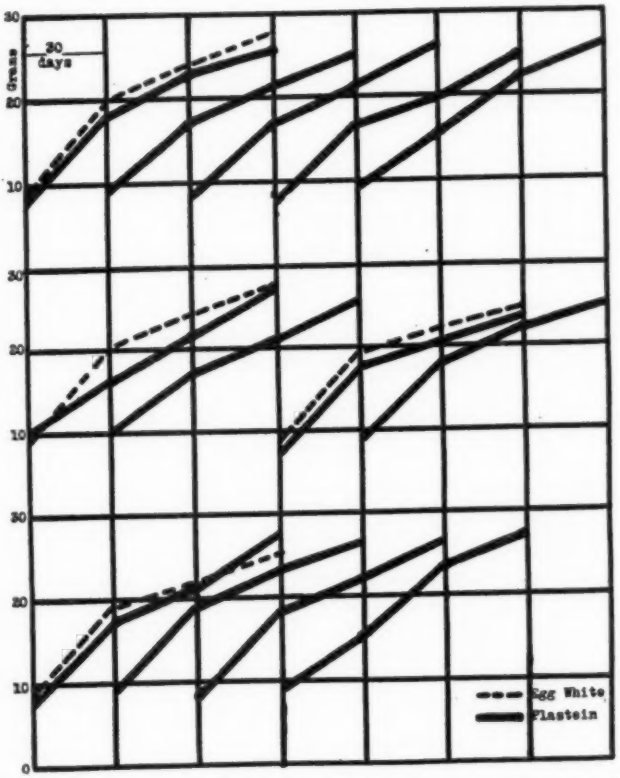

CHART 1. Comparison of the growth of female mice upon egg white and plastein as the sole source of protein in the diet.

of 3 months, it will be seen that there is at this time little difference in the growth, as revealed by body weight, between the two groups. In some cases the experimental animals made a better gain in weight than their controls in the same length of time.

As the preparation of the plastein was time-consuming, only fifteen animals were used in the experimental group. They were kept on the diet, however, for a period sufficient to demonstrate normal growth in the mouse (14). The hydrolytic products of the egg white were not fed as an additional control test, as it was very difficult to prepare this material in suitable condition for feeding. Furthermore, successful feeding trials with pepsin-digested egg white were not considered of any value in this investigation.

SUMMARY.

1. Plastein has been prepared from the hydrolytic products of commercial egg white by the method of Wasteneys and Borsook, who have shown chemically that this material is of the order of a native protein.

2. Feeding tests demonstrate that plastein from egg white digest satisfies the nitrogenous needs of the growing organism.

3. It is also shown that plastein can replace egg white as the sole protein in the diet of mice.

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ON THE NATURE OF THE URINE SUGARS.

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INTRODUCTION.

In spite of an extensive literature, and numerous methods of attack, the nature of the urine sugars is still in doubt. Following the early work of Baisch (1), Pavy and Siau (22), Breul (8), and others, it had seemed clearly established that the normal daily output of urine contained from 0.3 to 1.9 gm. of non-nitrogenous reducing substances, of which glucose constituted from 30 to 60 per cent. That glucose was present seemed definitely proven by the optical rotating powers of urine, by its fermentation with yeast, with the production of carbon dioxide, by the formation of benzoyl glucose when urine was shaken with benzoyl chloride, and the formation of an osazone in all respects identical with glucosazone (1). The close agreement in the value for glucose as obtained by fermentation and polariscopic determinations seemed conclusive.

That the urine does contain non-nitrogenous reducing substances simulating sugars, is recognized by almost all recent workers (5, 11, 13, 15, 16, 18, 21). The concentration of these substances has been found to vary from 0.01 to 0.2 per cent, representing a daily excretion of from 0.25 to 1.6 gm. Whether even a portion of this is true glucose, however, has become very doubtful. For the optical rotation measured by Baisch (1) is of very small magnitude, and in view of the complexity of urine, it is impossible to ascribe such rotation to glucose. Höst (15) has been unable to form glucosazone by heating urine with phenylhydrazine, using a method sensitive to 0.05 per cent of glucose. Osazones are formed, it is true, but they differ in size, shape,

color, and arrangement from glucosazone. The formation of carbon dioxide upon incubating urine with yeast is negatived by Lund and Wolf (16).

Recently, however, Benedict (6) has found that the ingestion of carbohydrates is followed by an increased sugar output in the urine, and that the consumption of glucose causes a corresponding increase in the fermentable fraction of urine sugar. He has therefore suggested that the term glycosuria, implying the sudden appearance of a sugar hitherto absent, be replaced by the term glycuresis, signifying merely a quantitative change, an *increase* in sugar excretion. It is evident that he considers glucose normally present in urine, and glucuresis, an increase in glucose excretion following its ingestion, to be a physiological occurrence, a specific example of the more general term glycuresis. Accordingly, the sugar in diabetic urine would represent, not a pathological breakdown in an absolute tolerance, but only a quantitative exaggeration of a preexisting normal excretion. To safeguard the kidney, he proposes prophylactic limitation of carbohydrate intake even in normal people.

The validity of this conception has been greatly discussed within the last few years. Folin and Berglund (11) have taken a diametrically opposed position: there is a true glycuresis after every carbohydrate meal, consisting entirely of foreign carbohydrates present in grains, nuts, and fruits, and of decomposition products formed in process of preparing food. Glucose is normally not present in urine, and appears only when a certain critical level of tolerance has been exceeded. Glucose ingestion up to 200 gm. fails to break down this barrier and does not cause a perceptible increase in the excretion of urine sugar. In view of these findings they are inclined to discount the significance of yeast fermentation. Benedict (4), in an analysis of their data, calls attention to a slight increase in the excretion of urine sugars following glucose intake, and points out that such an increase is proof for the presence of glucose normally, and the validity of his concept of glycuresis. But Höst (15) as well as Greenwald, Gross, and Samet (13) also finds that the ingestion of glucose causes no increase in the excretion of urine sugar and both conclude that the so called urine sugars represent by products of metabolism. Yet Blatherwick and her coworkers (7) confirm Benedict's find-

ings, and ascribe the non-fermentable fraction of urine sugar to by products of protein metabolism.

The results of fermentation experiments are no less confusing, to a great extent due to lack of uniformity in method. Shaffer and Hartmann (23) report no decrease in sugars following incubation with yeast, but give no details as to method. Greenwald, Gross, and Samet (13) obtain variable results upon incubating at 37–38° for 24 hours, and place no reliance in the results obtained by fermentation methods. Most workers find a 20 to 70 per cent decrease in amount of reducing substances upon incubation with yeast, a decrease which is usually ascribed to the presence of glucose. Yet as already noted, Lund and Wolf (16) are unable to demonstrate formation of carbon dioxide, and Höst finds that while glucose is fermented under his conditions in 24 to 48 hours, the decrease in urine sugars is not completed in less than 48 to 72 hours. And very recently, Patterson (21), by the addition of toluene to the urine inhibits what she takes to be a bacterial decomposition, and obtains no fermentation under conditions in which glucose is completely fermented within 17 hours.

I. Scope of Present Investigation.

The present experiments grew out of some work upon blood sugar fermentation, in the course of which we were able to confirm the finding of Hiller, Linder, and Van Slyke (14) that, using proper methods, the fermentation of blood sugar by yeast is completed within 20 minutes. In the light of this finding, it was somewhat surprising to note that as far as could be ascertained, none of the previous work upon urine had involved fermentation for less than 17 hours, many workers fermenting urine for as long as days, by which time bacterial decomposition is quite evident. As will appear, we were able to show that any glucose present in urine might, under proper conditions, be completely fermented within 40 minutes. Using this method as the basis for glucose determinations, the effect of foods and especially of glucose upon the excretion of urine sugars was then investigated.

In these determinations, Benedict's recent copper and tungstate reagents (2) were used. They were found to yield consistent results over a wide range, the values for urine sugar being slightly

lower than those obtained by using the Folin-Wu reagents (12). The green color of the blank which makes their use impossible in blood fermentation, here, because of the lesser dilution (1:4 instead of 1:10) makes readings difficult only for solutions containing less than 0.01 per cent sugar. The new alkaline copper tartrate and molybdic acid reagents suggested by Folin (9) for use in blood and urine were found to be useful only for blood sugar determination. In making determinations of urine sugar by this method, fully 20 to 30 per cent of sugar added to urine is not recovered. An extensive series of determinations has shown that the loss is due to some substances dissolved from the Lloyd's reagent used, substances which, as Benedict has suggested, probably remove hydroxides and carbonates from solution when the alkaline reagent is added.[1]

II. Fermentation of Glucose in Pure Solution.

To each of a series of flasks were added 5 cc. of glucose solutions of varying concentration and 3 cc. of the yeast suspension described by Hiller and Van Slyke in blood sugar fermentation;[2] the mixture was shaken and incubated at 37–38° for varying intervals. Upon withdrawal from the incubator, each mixture was diluted to 20 cc. and shaken with Lloyd's alkaloidal reagent in order to secure a water-clear filtrate. Determinations were made upon 2 cc. of filtrate, using appropriate standards. The results are tabulated in Table I.

It is quite evident that glucose within the physiological range of concentration is completely fermented within 40 minutes. Two factors are of great importance in determining the speed of fermentation, and explain the failure of previous workers to obtain complete fermentation in less than 17 hours. The first of these is the use of inadequate quantities of yeast. The dilution used in

[1] In his last communication, published after this study had been completed (Folin, O., and Svedberg, A., *J. Biol. Chem.*, 1926, lxx, 405), Folin suggests a method of overcoming this difficulty.

[2] One cake of Fleischmann's yeast suspended in 20 cc. of water. There are thus obtained 32 cc. of suspension. The water content of a cake of yeast being 9 cc. (14, 17), 3 cc. of the suspension represent $\frac{3}{32} \times (20 + 9) = 2.72$ cc. of water. In the final dilution of 20 cc. this represents a percentage error of 1.4 per cent, which is well within limits of accuracy of the method.

the foregoing experiment corresponds to 16.4 per cent by weight of yeast cake.

None of the earlier workers (1, 8, 22) gives the exact quantity of yeast used. MacLean (17) simply mixed urine well with yeast. Oppler (20) used 1 cc. of yeast to 100 cc. of urine—a maximum of 0.63 per cent by weight of yeast cake. Höst (15) added yeast to 5 per cent, and Patterson (21) to 0.5 per cent. Benedict (3) and most of the subsequent workers (7, 13, 19, 24) have used quite sufficient quantities; *viz.*, $\frac{1}{4}$ cake of yeast to 20 cc. of urine, 17.5 per cent of yeast by weight. It is difficult to see why none of the workers who have been using yeast in quantities suggested by Benedict, a quantity sufficient for rapid fermentation, has commented on the discrepancy in the relative speeds of fermentation of glucose and of urine.

TABLE I.

Glucose is completely fermented in 40 minutes at 37–38°.

Glucose.	10 min.	20 min.	30 min.	40 min.
per cent				
0.020	0.005	0.005	0.003	0.003
0.049	0.003	0.003	0.003	0.003
0.100	0.030	0.016	0.003	0.003
0.400		0.160	0.025	0.003
Blank.	0.003	0.003	0.003	0.003

But just as important as the amount of yeast used is the volume of fluid incubated. For, within 10 to 15 minutes after being shaken, most of the yeast has settled as a compact layer in the bottom of the flask, covered by a relatively slightly cloudy suspension. The bulk of fermentation takes place in the lower layers of fluid to which yeast has gravitated. Obviously, in the large volumes of urine usually used, doubling the quantity of yeast will not speed up fermentation as much as halving the volume of fluid.

III. Fermentation of Urine Sugar.

When the method used for pure glucose solutions was applied to urine, however, difficulty was encountered. 5 cc. of urine were shaken with 3 cc. of the yeast suspension and incubated at

37-38°. Following incubation there were added 7 cc. of water and 5 cc. of 0.1 N H_2SO_4, and the mixture shaken with 1.5 gm. of Lloyd's alkaloidal reagent for 2 minutes, a procedure suggested by Folin for the removal of most of the interfering substances in urine (10). As appears in Table II, far from an immediate decrease, there is a gradual increase in the amount of reducing substances extending over several hours, followed by an even slower decrease over several days. It is this decrease which most workers have ascribed to a true fermentation of glucose. Yet, confused as results were by this initial increase due to a puzzling

TABLE II.

Incubation of urine with yeast leads to slow formation of reducing substances, obscuring the fact that glucose is completely fermented within 30 to 40 minutes, with a subsequent slow decrease over 48 hours.

Reducing substances.	20 min.	40 min.	2 hrs.	24 hrs.	48 hrs.
per cent					
1. 0.068	0.090	0.121	0.110	0.082	0.050
1′. 0.264	0.120	0.093	0.105	0.070	
2. 0.022	0.030	0.056	0.070	0.015	0.005
2″. 0.123	0.032	0.060	0.072	0.020	0.003
3. 0.084	0.094	0.106	0.094	0.030	0.060
4. 0.065	0.073	0.070	0.060	0.020	0.020
5. 0.028	0.033	0.032	0.024	0.020	0.029
Blank.	0.003	0.003	0.003	0.005	0.005

1′ = 1 + 0.200 per cent glucose.
2″ = 1 + 0.100 " " "

reaction between urine and yeast, it was apparent that any glucose added to urine was completely fermented within 40 minutes, as shown in Table II.

It was obviously impossible to decide upon the presence or absence of glucose until this extensive formation of reducing substances could be eliminated. A pH to either side of the neutral point of phenolphthalein retarded, but did not prevent, the urine-yeast reaction. It was finally found that if the urine was shaken with Lloyd's reagent preliminary to fermentation, those substances in the urine responsible for the confusing reaction were entirely removed.

To 5 cc. of urine were added 10 cc. of water and 5 cc. of 0.1 N sulfuric acid and the mixture shaken with 1.5 gm. of Lloyd's reagent (Folin (10)). To 10 cc. of the 13 to 14 cc. of filtrate thus obtained were added 3 cc. of yeast suspension and the whole incubated at 37-38°. Determinations were made upon 2 cc. of filtrate from the mixture. As before, the addition of 0.5 gm. of Lloyd's reagent prevents the passage of yeast through the filter paper. To allow for the dilution with yeast suspension, the results obtained are multiplied by 1.27 (3 cc. of yeast suspension = 2.72 cc. of water).

TABLE III.

No fermentation occurs in the filtrates from urines shaken with Lloyd's alkaloidal reagent, although glucose added is quantitatively fermented within 40 minutes.

Reducing substances.	20 min.	40 min.	2 hrs.	48 hrs.
per cent				
1. 0.033	0.033	0.035	0.034	0.006
1′. 0.235	0.048	0.037	0.036	0.006
2. 0.055	0.052	0.057	0.059	0.030
2″. 0.152	0.063	0.056	0.057	0.035
3. 0.089	0.092	0.090	0.090	0.050
4. 0.075	0.080	0.078	0.074	0.032
5. 0.044	0.041	0.046	0.045	0.035
Blank. 0.003	0.003	0.003	0.003	0.005

1′ = 1 + 0.200 per cent glucose.
2″ = 1 + 0.100 " " "

It is evident from Table III that the preliminary shaking with Lloyd's reagent has removed from urine whatever substance was responsible for the production of reducing substances upon incubation with yeast. There is now found an extremely slow decrease in apparent value of urine sugar over a period of days, a decrease which cannot be attributed to glucose fermentation, for any glucose added to urine is quantitatively fermented within 40 minutes. Yet the same urines which, after treatment with Lloyd's reagent, fail to show any fermentation in 40 minutes, will, when incubated directly with yeast, show a decrease of 20 to 70 per cent in 24 hours, as shown in Table IV. Such a decrease is,

therefore, not due to glucose fermentation, occurs even in the Lloyd's filtrate on prolonged incubation, and, as has been suggested by recent workers, is possibly due to bacterial decomposition.

IV. Effect of Ingestion of Pure Glucose upon Urine Sugar Excretion.

100 gm. of glucose in 500 cc. of water, flavored with coffee to prevent nausea, were given to eight patients, the last previous meal having been eaten on the preceding night, and blood obtained as in tolerance test, after ½ hour, 1 hour, and 2 hours. Urine was obtained just before glucose ingestion and every half hour

TABLE IV.

Changes in reducing value of urine upon incubation with yeast over long intervals do not represent true fermentation of glucose.

Urine.	Direct fermentation of urine with yeast.			Fermentation of Lloyd's filtrate.	
	40 min.	24 hrs.	48 hrs.	40 min.	24 hrs.
1. 40*	59	25	20	43	20
2. 85	96	44		84	60
3. 112	134	40	68	116	72
4. 67	93	21	15	65	38
5. 75	91	50		75	
6. 87	97	26	6	83	39
7. 41	65	12	19	44	27
8. 212	115	62	23	82	75
9. 374		80		92	83

* Figures represent mg. of reducing substances per 100 cc. of urine.

thereafter over several hours. To facilitate diuresis, 60 cc. of water were given by mouth every half hour. Of the subjects two were known diabetics, two were normal, and four were patients with chronic arthritis, none of whom, so far as was known, had any difficulty in metabolizing carbohydrates. Of the arthritic patients, two had normal blood sugar curves, and two showed examples of the so called "prediabetic" curve. The results are tabulated on the following pages.

In all the normal (and arthritic) patients, the fasting urine specimen was absolutely free of glucose. In some of these it

remained so throughout the experiment (Table V). In others (Table VI) no sugar appeared until a certain critical level of

TABLE V.

No appearance of glucose in urine after ingestion of 100 gm. of glucose by arthritic subject.

Blood.		Urine.						
				Sugar.				
Time.	Sugar.	Time.	Volume.	Total.	Non-fermentable.	Fermentable.	Non-fermentable.†	Fermentable.†
	per cent		*cc.*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
9.30	0.082	9.25	68*	0.084	0.082		28.5	
10.00	0.153	9.55	30	0.095	0.097		28.5	
10.30	0.183	10.25	30	0.053	0.050		15.9	
11.00	0.148	10.55	210	0.018	0.018		37.8	
11.30		11.25	120	0.020	0.019		24.8	
12.00	0.090	11.55	125	0.019	0.020		23.8	

* 2 hours excretion.
† Mg. excreted per half hour.
Glucose ingested at 9.31.

TABLE VI.

Temporary overflow of glucose into urine after ingestion of 100 gm. of glucose in normal subject.

Blood.		Urine.						
				Sugar.				
Time.	Sugar.	Time.	Volume.	Total.	Non-fermentable.	Fermentable.	Non-fermentable.*	Fermentable.*
	per cent		*cc.*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
10.16	0.082	10.19	200†	0.020	0.021		6.4	
10.46	0.126	10.49	33(?)	0.022	0.020		7.2	
11.30	0.102	11.19	48	0.103	0.026	0.075	12.5	36
		11.49	48	0.033	0.021	0.012	10.8	5.8
12.16	0.090	12.19	76	0.020	0.022		16.8	

* Excretion per half hour.
† 3 hours excretion.
Glucose taken at 10.17.

blood sugar was reached, whereupon there was a sudden appearance of fermentable sugar in the urine, in some cases in large quantities, in others only in traces. Following its initial appear-

ance, the excretion of glucose continued even after the blood sugar had fallen below its original critical level, but in all the patients, sugar had disappeared from the urine by the 3rd hour.

In the diabetic patients, glucose was found in urine even at the fasting level of blood sugar as shown in Table VII. It is to be remembered, however, that this is not necessarily true of diabetics, some of whom develop even an increased tolerance for sugar.

In most of the patients tested there was noted, during the 2nd hour after ingestion of glucose, an increase in the excretion of non-fermentable carbohydrate. Whether this is due to varia-

TABLE VII.

Constant overflow of glucose into urine before and after ingestion of 100 gm. of glucose by a diabetic patient.

Blood.		Urine.						
				Sugar.				
Time.	Sugar.	Time.	Volume.	Total.	Non-fermentable.	Fermentable.	Non-fermentable.*	Fermentable.*
	per cent		*cc.*	*per cent*	*per cent*	*per cent*	*mg.*	*mg.*
10.00	0.200	10.05	65	0.175	0.090	0.085	58.5	>200
10.30	0.275	10.35	50	>0.500	0.110	>0.500	55.0	>200
11.00	0.365	11.05	73	>0.500	(?)	>0.500	(?)	>200
		11.35	30	>0.500	0.080	>0.500	24.0	>200
12.00	0.372	12.05	85	>0.500	0.070	>0.500	59.6	>200

* Excretion per half hour.
Glucose taken at 10.02.

tions in the rate of urine secretion which become evident on taking specimens over short intervals of time, whether it is due to foreign carbohydrates supplied by the coffee used to flavor the sugar solution, or whether the mobilization of carbohydrates in blood affects the non-fermentable as well as the fermentable fraction, we are unable to say.

It is this increase which has led Benedict to believe that glucose is normally excreted in quantities which may be increased by its ingestion. A faulty method of fermentation apparently confirmed the fact. It has been seen, however, that the increase is altogether in the non-fermentable fraction, and can by no means be ascribed to glucose excretion.

V. Effect of Commercial Glucose and an Ordinary Meal upon Excretion of Urine Sugar.

Objections have been raised (4) to such experiments as the foregoing that the shock caused the organism by the ingestion of very large quantities of carbohydrates may well prevent a glucuresis which would occur from the feeding of more moderate quantities of glucose. To test this, two normal individuals took small amounts of commercial glucose (chocolate) as the first food taken in the morning. The figures do not include volume of excretion, but the qualitative results seem definite (Table VIII).

TABLE VIII.

Ingestion of commercial glucose by normal subjects results in an increased excretion of non-fermentable reducing substances, but not of glucose unless tolerance has been exceeded.

	Sugar.		
	Total.	Non-fermentable.	Fermentable.
	per cent	*per cent*	*per cent*
a. Fasting	0.019	0.020	
b. 10 gm. of glucose	0.028	0.027	
a. Fasting	0.033	0.036	
b. 30 gm. of glucose	0.085	0.088	
a. Fasting	0.036	0.034	
b. 20 gm. of glucose	0.053	0.051	
a. Fasting	0.045	0.047	
b. 85 gm. of glucose	0.110	0.090	0.025

a, collected 15 minutes before taking glucose.
b, collected approximately 1½ to 2 hours after taking glucose.

In only one case did glucose at any time appear in the urine, and this was after the ingestion of a quantity of glucose which may well have exceeded the tolerance of that individual. The marked increase in the concentration of non-fermentable reducing substances after the ingestion of the chocolate is too uniform to be an accident of secretion and is probably due to the non-glucose carbohydrates present in the material ingested.

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TABLE IX.

Ingestion of an ordinary meal by normal subjects results in an increased output of non-fermentable reducing substances, but *not* of glucose.

	Sugar.		
	Total.	Non-fermentable.	Fermentable.
	per cent	*per cent*	*per cent*
a. Before meal	0.019	0.022	
b. After "	0.084	0.080	
a. Before "	0.044	0.042	
b. After "	0.100	0.104	
a. Before "	0.035	0.031	
b. After "	0.075	0.078	

a, collected 10 minutes before meal.
b, collected approximately 1 to 2 hours after meal.

TABLE X.

In the non-fasting subject, the ingestion of commercial glucose or a miscellaneous meal does not cause appearance of glucose in the urine.

	Total urine sugars.	Non-fermentable urine sugars.
	per cent	*per cent*
1. Before meal	0.053	0.055
After "	0.100*	0.098
2. Before "	0.020	0.017
After "	0.057*	0.052
3. Before "	0.036	0.037
After "	0.078*	0.083
4. Before 50 gm. of glucose	0.059	0.063
After 50 " " "	0.068*	0.064
5. Before 50 " " "	0.083	0.080
After 50 " " "	0.047*	0.043

* Since volume of urine secretion was not measured, these figures represent qualitative changes only.

In six normal individuals, following an ordinary mixed meal including bread, meat, milk, potatoes, and fruit in varying quantities and combinations, there was a marked increase in the excretion of non-glucose reducing substances, both in concentration and total output. But in no case was fermentable sugar present either before or after the ingestion of food (Table IX).

The threshold tolerance for glucose, indicated by the foregoing experiments, is not peculiar to the fasting state. The ingestion of commercial glucose and of a miscellaneous meal by non-fasting individuals approximately 5 hours after the preceding meal, gives results qualitatively similar to those obtained in fasting subjects.

SUMMARY AND CONCLUSIONS.

By incubating urine at 37-38° with appropriate quantities of yeast, any glucose present up to 0.4 per cent may be quantitatively fermented within 40 minutes. The presence of glucose in concentrations as low as 0.010 per cent may thus be determined, provided certain substances in urine which obscure the results of fermentation are first removed. Lloyd's alkaloidal reagent, suggested by Folin for use in determination of urine sugar, has been found to be useful also in this regard.

Using this method, it is found that glucose is not normally excreted in the urine. What has heretofore been considered to be glucose, fermentable by yeast, is in reality a group of substances which only gradually decompose under the conditions of fermentation, possibly due to bacterial decomposition. Such decomposition is a matter of days, while the fermentation of glucose is completed in less than an hour. It is these substances which are partially removed by the preliminary shaking of urine with Lloyd's reagent.

Until a certain critical level of blood sugar has been reached, the kidney interposes an absolute barrier against the excretion of glucose. The ingestion of moderate quantities of glucose by the normal individual fails to exceed this threshold value, and is accordingly not followed by the appearance of even traces of glucose in the urine. It is only when such large quantities as 100 gm. of glucose are taken that this critical level of blood sugar is exceeded in a certain proportion of normal individuals, and then only does fermentable sugar appear in the urine. Once

begun, however, the excretion of glucose continues even after the blood sugar has receded to below this level.

It is quite doubtful whether the normal individual, on an average diet, ever shows true glucose in the urine. The increase in urine sugar following food (and glucose) intake, discovered by Benedict and termed by him glycuresis, represents, not glucose, but non-fermentable substances. In the case of food it is probably true, as has been suggested, that these are either foreign non-assimilable carbohydrates originally present in the food, or decomposition products formed in the process of preparing food or of its digestion. The increase in their excretion following the ingestion of glucose is somewhat difficult to understand, and may be due to any of several factors suggested: variations in the rate of secretion, a trace of impurities in the glucose used, or a mobilization of these substances in the blood coincident with the rise in blood sugar.

I wish to express my thanks to Dr. W. A. Perlzweig, whose kind advice and cooperation at every turn have made this investigation possible.

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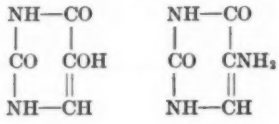
THE OXIDATION OF 5-AMINOURACIL.

By OSKAR BAUDISCH and DAVID DAVIDSON.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 16, 1926.)

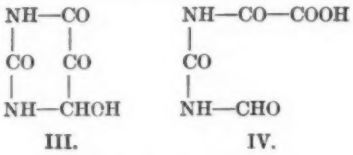
The results obtained by the authors in the oxidation of isobarbituric acid I[1] by means of ferricyanide have led them to a study



I. II.

of the behavior of this reagent toward other 5-substituted pyrimidines, of which 5-aminouracil II will be discussed in this paper.

Earlier investigators have shown that bromine water converts 5-aminouracil into isodialuric acid III[2] while permanganate splits



III. IV.

the ring producing formyl-oxalylurea IV.[3] As in the case of isobarbituric acid[1] ferricyanide here too yields a unique oxidation product.

It was anticipated that just as the corresponding hydroxy compound (isobarbituric acid I) yields diisobarbituric acid V

[1] Davidson, D., and Baudisch, O., *J. Biol. Chem.*, 1925, lxiv, 619.
[2] Behrend, R., and Roosen, O., *Ann. Chem.*, 1889, ccli, 235.
[3] Offe, G., *Ann. Chem.*, 1907, ccccliii, 278.

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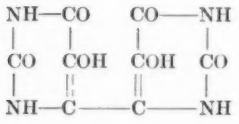
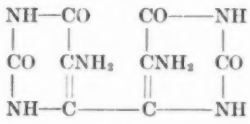


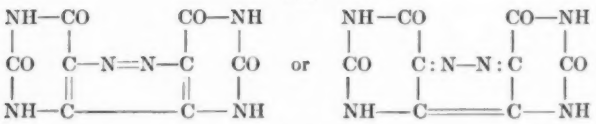
V.



VI.

so 5-aminouracil II might yield 5,5′-diaminodiuracil. The reaction probably proceeds through this stage but analysis of the reaction product leads to the conclusion that oxidation continues further. If initial linking of the 4 carbons of two aminouracil rings be assumed, the ferricyanide could then act in its characteristic manner[4] upon the two juxtaposed amino groups and thereby produce a double bond between the nitrogen atoms involved. Thus a third cycle—the pyridazine ring VII—would be formed and there would result the tricyclic diuracil pyridazine VIII.

VII.



VIII.

EXPERIMENTAL PART.

5-Aminouracil was prepared by the method of Johnson and Matsuo[5] from 5-nitrouracil.[6]

Diuracil Pyridazine.—A chilled, filtered solution of 38.8 gm. of potassium ferricyanide in 250 cc. of water was gradually added with mechanical stirring to a chilled, filtered solution of 8.9 gm. of

[4] Bamberger, E., and von Goldberger, A., *Ber. chem. Ges.*, 1898, xxxi, 2639.
[5] Johnson, T. B., and Matsuo, I., *J. Am. Chem. Soc.*, 1919, xli, 782.
[6] Davidson, D., and Baudisch, O., *J. Am. Chem. Soc.*, 1926, xlviii, 2379.

potassium hydroxide and 5 gm. of 5-aminouracil in 150 cc. of water. Almost at once precipitation of the brick-red potassium salt of diuracil pyridazine began. Complete precipitation of the product was secured by stirring for a few minutes after all the ferricyanide had been added. After passing in CO_2 to insure absence of alkali the reaction mixture was centrifugalized, the product washed by suspending in 1 per cent KCl and centrifugalizing again. After a second washing, the salt was suspended in alcohol, filtered, and washed with alcohol. In this manner, the product was conveniently obtained as a fine powder. From a dilute solution of the potassium salt, silver nitrate precipitates a deep wine-colored silver salt.

To obtain the free pyridazine, the potassium salt was suspended in water and poured into 200 cc. of hot water containing an excess of hydrochloric acid. The highly insoluble diuracil pyridazine separated immediately in orange-yellow diamond-shaped platelets. On cooling, the material was centrifugalized and then washed with water and alcohol as in the case of the potassium salt. Yield 2.5 gm. (51 per cent of theory).

Analysis.

$C_8H_4O_4N_6$.	Calculated.	C 38.69, H 1.63, N 33.88.
	Found.	" 38.74, " 1.84, " 33.66.

Diuracil pyridazine does not melt below 300°. It is insoluble in common organic solvents, very sparingly soluble in water, soluble in concentrated H_2SO_4. When strongly heated in a tube it decomposes while a reddish yellow sublimate is produced, which dissolves in organic solvents and water to give strongly fluorescent solutions. It is unattacked by concentrated nitric acid or bromine water. With sodium hydroxide it at first forms the insoluble red sodium salt but dissolves in excess of alkali to form a yellow solution with beautiful orange fluorescence.

CATALYTIC OXIDATION BY MEANS OF COMPLEX IRON SALTS.

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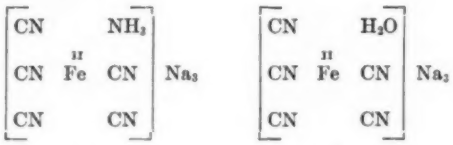
(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

(Received for publication, November 16, 1926.)

The observation of Manchot[1] that sodium pentacyano-ammine-ferroate[2] I absorbs oxygen has led Baudisch and his coworkers[3] to study the catalytic action of this salt, as well as of its water analogue II, in the oxidation of pyrimidines and purines. The present authors have previously reported in this journal[4] on the nature of the oxidation products obtained. In the present paper the problem is considered from the standpoint of the catalyst. The work has been limited to sodium-pentacyano-ammine-ferroate I because of the ready availability of this salt in a satisfactory state of purity.



I. II.

Theoretical.

In the system: water, sodium pentacyano-ammine-ferroate, oxygen, organic molecule, the state of affairs may be represented by the following equations.

[1] Manchot, W., *Ber. chem. Ges.*, 1912, xlv, 369.

[2] Hofmann, K. A., *Ann. Chem.*, 1900, cccxii, 1.

[3] Pfaltz, M. H., and Baudisch, O., *J. Am. Chem. Soc.*, 1923, xlv, 2972. Pfaltz, M. H., *J. Am. Chem. Soc.*, 1923, xlv, 2980. Baudisch, O., and Bass, L. W., *J. Am. Chem. Soc.*, 1924, xlvi, 184.

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$$H_2O \rightleftarrows H^+ + OH^- \tag{1}$$

$$\begin{array}{c} \tfrac{1}{2}O_2\ (\text{Gas}) \\ \downarrow\uparrow \\ H^+ + \tfrac{1}{2}O_2\ (\text{Dissolved}) + 2e \end{array} \rightleftarrows OH^- \tag{2}$$

$$Na_3[FeCN_5 \cdot NH_3] \rightleftarrows [FeCN_5 \cdot NH_3]^{---} + 3Na^+ \tag{3}$$

$$[FeCN_5 \cdot NH_3]^{---} \rightleftarrows [FeCN_5 \cdot NH_3]^{--} + e \tag{4}$$

$$\begin{array}{c} \tfrac{1}{2}O_2\ (\text{Gas}) \\ \downarrow\uparrow \\ (2) + (4)\quad H^+ + \tfrac{1}{2}O_2\ (\text{Dissolved}) + 2[FeCN_5 \cdot NH_3]^{---} \end{array} \rightleftarrows OH^- + 2[FeCN_5 \cdot NH_3]^{--} \tag{5}$$

Equation 2 represents the tendency of hydrogen ion to react with dissolved oxygen, taking up 2 electrons to form hydroxyl ion. Equation 4 indicates the tendency of the complex ferroate ion to give up 1 electron to become the ferriate ion, while Equation 5, which combines Equations 2 and 4, sums up the reactions involved. Though Equation 5 is actually not reversible yet it becomes so in the presence of an oxidizable organic compound such as a pyrimidine, which can act as an acceptor for the oxygen theoretically capable of being liberated. Since, moreover, the oxidation of the pyrimidine is certainly not reversible, the ferriate ion produced by the forward process of Equation 5 is continuously reduced to the ferroate ion by the pyrimidine present so that the complex ion, therefore, acts as an oxygen carrier or oxidation catalyst.[5]

Apparatus.

The course of the catalyzed oxidations represented in the accompanying charts was followed by measuring the oxygen absorbed. The Van Slyke amino apparatus[6] was adapted to this purpose. At the beginning of an experiment the apparatus was completely filled with water. By adjusting the stop-cocks properly the excess of water in vessel *A* was allowed to run out. The leveling bulb was lowered and about 65 cc. of oxygen passed

[5] In this connection see Smith, J. H. C., and Spoehr, H. A., *J. Am. Chem. Soc.*, 1926, xlviii, 107.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1912, xii, 278.

from a reservoir into burette F through stop-cock c, which was then turned, and the leveling bulb raised above the apparatus. The compressed gas in the burette was now allowed to force out through stop-cock d the water contained in the vessel D. By this means the water originally in the apparatus was displaced by oxygen. Lowering the leveling bulb until the gas in the apparatus was below atmospheric pressure allowed solutions or suspensions to be drawn into the deaminizer D from its burette B. The volume of D having previously been found, the total volume of gas in the apparatus at any moment during an experiment was determined by reading the level of the water in burette F.

In the experiments described the volume of solution used was 20 cc. A suspension of the organic compound in 10 cc. of buffer solution was first introduced, followed by 10 cc. of a solution of proper concentration of the iron salt, prepared just before use in an evacuated test-tube. The shaker was operated at 500 strokes per minute. The temperature was 20°C. ($\pm 2°$) and the pressure 760 mm. (± 10 mm.). The volumes recorded in the figures are of moist oxygen reduced to standard conditions.

The buffer solutions employed were made up so that each 100 cc. contained: for pH 2, 9.8 gm. of H_3PO_4 + 9.1 gm. of KH_2PO_4; for pH 7, 7.1 gm. of Na_2HPO_4 + 4.5 gm. of KH_2PO_4; for pH 12, 9.5 gm. of Na_3PO_4 + 0.4 gm. of KH_2PO_4.

DISCUSSION OF RESULTS.

As is seen from Equation 5 the oxidation of the pentacyano-ammine-ferroate ion by elementary oxygen involves hydrogen ion. It is, therefore, to be expected that the rate of oxidation of the complex ion will be dependent on the hydrogen ion concentration. That this rate is actually inversely proportional to the pH of the solution is seen in Fig. 1.

It is also a consequence of Equation 5 that aside from other considerations the rate of reduction of the complex ferriate ion will be dependent on the hydroxyl ion concentration; that is to say, directly proportional to the pH. The ability of the pentacyano-ammine-ferroate ion to act as an oxygen carrier depends not only on the rate of oxidation of the complex ferroate ion by elementary oxygen, but also on the rate of reduction (in the presence of some

organic compound) of the complex ferriate ion (essentially the oxidation potential of the ferriate-ferroate mixture). Since these two rates are *oppositely* affected by the pH of the solution, it follows that what might be called the rate of oxygen carriage will

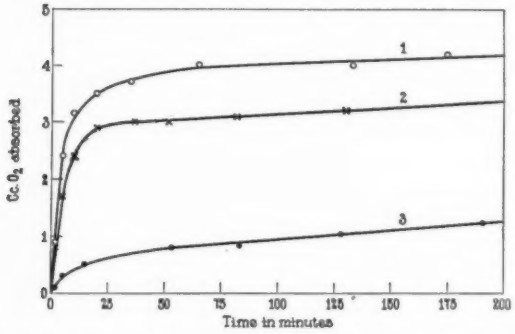


FIG. 1. The rate of oxygen absorption by the pentacyano-ammine-ferroate ion is inversely proportional to the pH of the solution.

In each experiment, 1 millimol of the complex iron salt was dissolved in 20 cc. of buffer solution. Curve 1, pH 2; Curve 2, pH 7; Curve 3, pH 12.

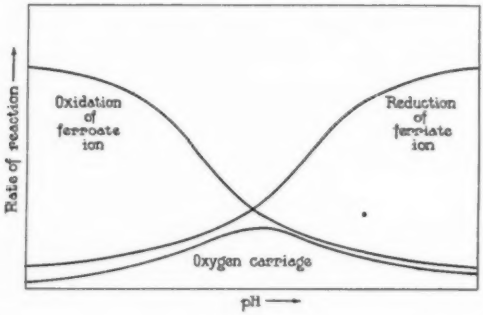


FIG. 2.

have a *maximum* at some pH from which it will fall off on either side. These relations may be illustrated schematically as in Fig. 2.

Besides the variation of oxygen carriage power with pH must also be considered the change of oxidizability or the "apparent

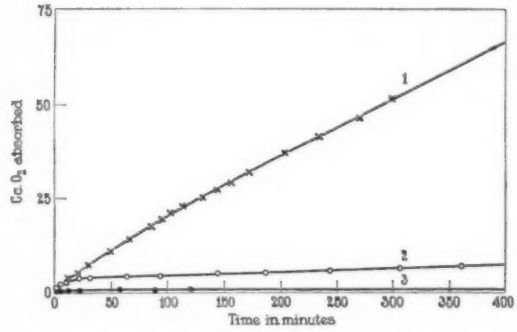


FIG. 3. The catalyzed oxidation of hydantoin is more rapid at pH 12 than at pH 7.

In each experiment, 10 millimols of hydantoin were suspended in 20 cc. of buffer solution containing 1 millimol of sodium pentacyano-ammine-ferroate. Curve 1, pH 12; Curve 2, pH 7; Curve 3, pH 12 (without catalyst).

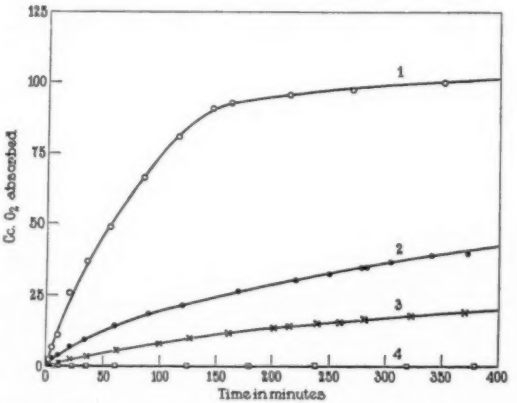


FIG. 4. The catalyzed oxidation of isobarbituric acid is more rapid at pH 12 than at pH 7.

In these experiments, 5 millimols of the pyrimidine were suspended in 20 cc. of buffer solution containing 1 millimol of catalyst. Curve 1, pH 1; Curve 2, pH 7; Curve 3, pH 12 (without catalyst); Curve 4, pH 7 (without catalyst).

oxidation potential"[7] of the organic compound. At all events the pH at which oxygen carriage to a given organic compound may be optimum will not necessarily be that at which the amount of oxygen absorbed by the pentacyano-ammine-ferroate ion itself is large as it is, for example, in acid solution, but may actually occur in the alkaline range where the oxygen absorption of the complex salt itself is relatively low. As a matter of fact in the investigated cases of hydantoin III[8] (Fig. 3), isobarbituric acid

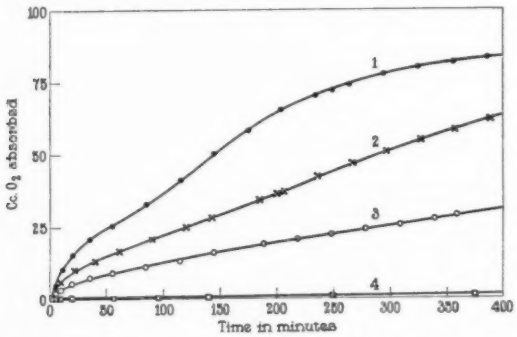
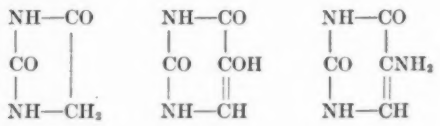


FIG. 5. The rate of catalytic oxidation of 5-aminouracil is proportional to the concentration of catalyst (pH 12).

In each experiment 5 millimols of the pyrimidine were suspended in 20 cc. of buffer solution which contained a varying amount of complex salt as follows. Curve 1, 2 millimols; Curve 2, 1 millimol; Curve 3, ½ millimol.

IV[9] (Fig. 4) and aminouracil V,[10] the optimum is somewhere on the alkaline side, since the rate of catalyzed oxidation is greater at pH 12 than it is at pH 7.



III. IV. V.

[7] Conant, J. B., *Chem. Rev.*, 1926, iii, 23.
[8] Harries, C., and Weiss, M., *Ann. Chem.*, 1903, cccxxvii, 369.
[9] Davidson, D., and Baudisch, O., *J. Biol. Chem.*, 1925, lxiv, 619.
[10] Baudisch, O., and Davidson, D., *J. Biol. Chem.*, 1927, lxxi, 497.

From Equation 5 it may be seen that the rate of oxygen carriage will also be dependent on the concentration of the complex ion. This relation is amply illustrated in the case of the oxidation of 5-aminouracil represented in Fig. 5.

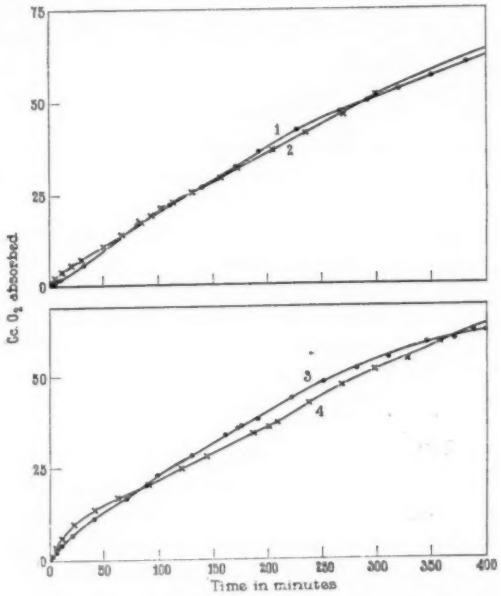


FIG. 6. Pentacyano-ammine-ferriate and ferroate are equally efficacious as oxygen carriers (pH 12).

Curve 1, 10 millimols of hydantoin with 1 millimol of ferrous salt. Curve 2, 10 millimols of hydantoin with 1 millimol of ferric salt: Curve 3, 5 millimols of 5-aminouracil with 1 millimol of ferrous salt. Curve 4, 5 millimols of 5-aminouracil with 1 millimol of ferric salt.

As previously stated Equation 5 is reversible only when an oxidizable organic compound is present to absorb the oxygen involved. This is borne out experimentally by the fact that when sodium pentacyano-ammine-*ferriate* is tested, as the corresponding ferrous salt previously was in pH 2, 7, and 12 (see Fig. 1) it *neither evolves nor absorbs oxygen.*

Since the cycle of reactions postulated above for the mechanism of the oxygen carrier action of sodium pentacyano-ammine-ferroate involves, in one stage, the reduction of the complex ferriate ion (Equation 5), essentially the same catalytic action should be exhibited by the ferriate salt as by the ferroate salt. That this condition is fulfilled is demonstrated in Fig. 6 in the cases of hydantoin and 5-aminouracil.

SUMMARY.

1. Pentacyano-ammine-ferroate ion is oxidized by elementary oxygen at a rate which is inversely proportional to the pH of the solution.

2. In the cases studied the rate of catalyzed oxidation is greater in alkaline solution than in neutral.

3. In the cases studied pentacyano-ammine-ferroate and pentacyano-ammine-ferriate ions have identical catalytic activity.

A METHOD FOR THE DETERMINATION OF MINUTE AMOUNTS OF ETHYL IODIDE IN AIR, WATER, AND BLOOD BY MEANS OF ITS REACTION WITH SILVER NITRATE; AND EXPERIMENTS BEARING ON THE DETERMINATION OF BLOOD FLOW BY MEANS OF ETHYL IODIDE.

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The ethyl iodide method of Henderson and Haggard (1) for measuring the cardiac output seemed to provide new and more convenient means for investigating this cardinal problem of the circulation in patients.

After having received instruction in the use of the method from its authors we proceeded to apply it to ourselves under conditions of basal metabolism. The results we obtained were similar to those of Henderson and Haggard and exhibited a uniformity at least as great as that obtained by other blood flow methods.[1] But we

[1] Blood flow determinations were made on two normal subjects by the ethyl iodide method modified as follows: (1) Rubber minimized. (2) Spirometer loaded with the same concentration (1.2 cc. of ethyl iodide in 300 liters) each day. (3) Bohr gas meter inserted in the alveolar circuit and the flow of air kept constant at approximately 0.2 liters per minute. (4) Flow through the iodine pentoxide train kept constant at approximately 1 liter per minute by a mercury valve which regulated the suction. (5) Two or more absorbers to catch the liberated iodine.

The subjects had had no food for 16 hours before the experiment and lay down for at least ½ hour before the first determination. Two or three determinations were made each day. These were separated by an interval of about 15 minutes during which the subject breathed no ethyl iodide and continued to recline. The Krogh-Henderson alveolar air averaged 5.2 per cent CO_2 for S, 5.4 per cent CO_2 for G. The pulse rate averaged 78 for S, 60 for G, and did not vary materially. G weighs 68 kilos, S weighs 77 kilos.

The results on G: Oct. 10, 4.2, 4.3, 4.2. Oct. 14, 5.3, 5.0. Oct. 20, 4.8, 5.4,

soon had cause to fear that the iodine pentoxide method for the determination of ethyl iodide was not reliable and to doubt the correctness of the physiology involved in the blood flow determination. Therefore we do not attach any significance to the blood flow figures we obtained, but their constancy encouraged us to continue the investigation.

The purpose of this paper is to describe chemical methods which appear adequate to attack the problem and to report the results of applying them to certain questions fundamental to the determination of blood flow by ethyl iodide. A micro method for the determination of ethyl iodide by means of its reaction with silver nitrate has been devised by one of us (S). By this means we have performed experiments on the rate of destruction of ethyl iodide in blood and in the body and have redetermined the coefficient of distribution of ethyl iodide in vapor and water, and in vapor and blood *in vitro*. The results differ from those obtained by previous investigators and throw doubt on the validity of the ethyl iodide blood flow method in its present form.

Iodine Pentoxide Method.

This method (2), originally devised for the analysis of carbon monoxide, consists of drawing ethyl iodide vapor through a heated U-tube containing iodine pentoxide, catching and titrating the liberated iodine. Henderson and Haggard originally directed that the flow must be rapid (at least a liter per minute),[2] and gave the equation:

$$10C_2H_5I + 13I_2O_5 = 20CO_2 + 25H_2O + 36I.$$

4.7. Oct. 26, 4.7, 4.8, 4,6. Oct. 29, 4.8, 5.7, 5.2. Nov. 9, 5.2. Nov. 16, 4.4, 4.8, 4.5. Eighteen determinations on subject G average 4.8 liters per minute; average deviation from the mean 6.7 per cent, maximum deviation 19 per cent.

The results on S: Oct. 12, 6.5, 6.1, 6.0. Oct. 13, 6.5, 6.4, 5.6. Oct. 19, 6.5, 6.0, 6.3. Oct. 21, 6.3, 6.3. Oct. 23, 5.9, 5.8. Nov. 2, 6.2, 5.8. Nov. 17, 6.1. Sixteen determinations on subject S between ½ and 2½ hours; S became restless after lying 2½ hours, average 6.1; average deviation from the mean 4.0 per cent, maximum deviation 8.2 per cent.

[2] At a demonstration at the International Congress of Physiology in August, 1926, Professor Henderson used a slow flow, and has recently described this technique (3).

It soon became apparent that with a rapid flow we obtained a yield of iodine far below the theoretical. The fact that a very slow flow yielded close to theoretical figures was first pointed out to us by Dr. A. V. Bock. Table I shows the influence of the

TABLE I.

Iodine Pentoxide Method.

Effect of Different Rates of Flow on Iodine Yield.

Date.	Iodine pentoxide tube.	Flow through iodine pentoxide train.	Ethyl iodide added.	Iodine recovered.	Iodine expected from Henderson-Haggard equation.	Per cent of theoretical recovered.
		liters per mm.	*mg.*	*mg.*	*mg.*	
Feb. 10	A	1.40	4.86	6.62	14.23	46.5
" 3(a)	"	0.99	3.78	5.77	11.08	52.1
" 3(b)	"	0.92	2.38	3.71	6.97	53.2
" 2	"	0.85	1.61	2.35	4.72	49.8
" 9	"	0.26	2.95	4.95	8.64	57.3
" 4	"	0.08	4.78	15.68	14.00	112.0
" 6	"	0.06	2.64	7.11	7.73	92.0
" 5	"	0.03	2.91	8.16	8.53	95.7
Mar. 8(a)	B	0.90	3.33	7.25	9.75	74.4
" 10*	"	0.75	1.87	4.12	5.48	75.0
" 9*	"	0.50	2.59	6.56	7.59	86.5
" 8(b)*	"	0.13	3.09	8.45	9.05	93.3

Tube A had been in use 7 months, B was a new tube. Both were obtained from Professors Henderson and Haggard.

The ethyl iodide was weighed in sealed bulbs, volatilized by breaking the bulb, and the vapor added to the air flowing through the train over a period of 10 minutes or more. Two absorbers were essential to catch the iodine, a third was always blank except in the experiments of Feb. 10, when it contained a minute trace. The thiosulfate was standardized by Kendall's method.

* In these experiments the vapor was preheated by passage through a glass coil immersed in the hot oil bath before its entrance into the iodine pentoxide tube. A comparison of Mar. 8(a) and Mar. 10 shows that this did not increase the yield.

rate of flow on the iodine yield of our two tubes. These results suggested that the rapid flow pulled a large part of the ethyl iodide through the iodine pentoxide unchanged. An experiment was made to test the point.

A preliminary experiment showed that ethyl iodide vapor could be bubbled through absorbers containing 30 cc. of 5 per cent KI without loss. A train was set up leading from outdoor air through one iodine pentoxide U-tube, through three absorbers each containing 10 cc. of 5 per cent KI, to the other iodine pentoxide tube, through two similar absorbers, through the gas meter to the suction pump. Ethyl iodide which escaped decomposition in the first tube would pass through the absorbers and liberate iodine from the second tube, while the iodine liberated from the first tube would be caught in the absorbers between them. As the amount of iodine liberated from each tube by a weighed amount

TABLE II.

Iodine Pentoxide Method.

Maximum Escape of Ethyl Iodide through Iodine Pentoxide Tubes.

Date.	Ethyl iodide added.	Flow.	Tube placed first.	Per cent of theoretical yield of iodine recovered from first tube.	Approximate per cent of ethyl iodide passing through first tube as determined by second tube.
	mg.	*liters per min.*			
Mar. 10	5.94	0.6	B	82.0	1.3
" 10	1.87	0.8	"	75.0	4.8
Apr. 15	4.18	0.95	A	53.8	7.7

The ethyl iodide added in the last two experiments was weighed; in the first, calculated from the iodine yield. Theoretical yield calculated from equation given by Henderson and Haggard.

of ethyl iodide at various rates of flow was known (Table I), the amount of ethyl iodide escaping through the first tube could be approximately calculated.[3] The results are given in Table II. Obviously the low yield is not caused by pulling ethyl iodide through the U-tube unchanged. Evidently the equation given above does not represent the chemical reaction when the flow is rapid.

Our difficulties with the method may be summarized as follows: (1) Different tubes of iodine pentoxide give different yields of

[3] This figure represents the maximum possible ethyl iodide. It is conceivable that decomposition products of ethyl iodide could escape from the first tube and liberate iodine from the second.

iodine under apparently similar conditions. (2) Variations in rate of flow make profound differences in iodine yield. (3) Iodine pentoxide is decomposed by traces of a large number of substances (4). (4) The spontaneous rate of decomposition of iodine pentoxide is quite variable. The proper correction for this decomposition is often doubtful. (5) While the method usually yields consistent results (within 4 per cent), occasionally it fails to do this, and we are not yet confident that we have all the factors under control.

Were it not for the fact that many of the errors cancel out in calculating the blood flow, the method could not be used in the simple form described, but would be difficult and time-consuming. We doubted our ability to overcome these difficulties and decided that further progress depended on the development of a more satisfactory chemical method.

Silver Nitrate Method for Determination of Ethyl Iodide in Air.

The method consists of adding standard silver nitrate in nitric acid to sampling tubes containing ethyl iodide, allowing the reaction to go to completion, and titrating the excess silver nitrate according to the Volhard principle.[4]

Silver nitrate and nitric acid in a closed tube are used as a step in the analysis of organic material for halogens by the Carius method. The reaction of ethyl iodide and silver nitrate in alcohol and hydro-alcoholic mixtures has been studied by Burke and Donnan (5). It goes to completion. In aqueous solution the reaction is formulated (6).

$$\text{I. } C_2H_5I + AgNO_3 + HOH = C_2H_5OH + AgI + HNO_3$$
$$\text{II. } C_2H_5I + AgNO_3 = AgI + C_2H_5NO_3$$

We have found that a high concentration of nitric acid accelerates the conversion of ethyl iodide vapor into silver iodide. Under these conditions reaction I is probably minimized and the organic radicals oxidized.

[4] An attempt to find a substance which would remove ethyl iodide from air bubbled through it was not successful. Cold alcohol was the best. We also failed in an attempt to determine ethyl iodide by pulling the vapor through a combustion furnace and catching the liberated iodine. Yields of over 90 per cent of the theoretical were not obtained.

XUM

Apparatus.—250 cc. gas sampling tubes are employed,[5] each end closed by black rubber tubing, pinch-cocks, and glass plugs. Long test-tubes, narrow enough to fit closely inside the glass end of the sampling tube, are used, or wider tubes ground to make a tight joint with this end.

Reagents Required.—(1) Silver nitrate solution in concentrated nitric acid (2.1785 gm. per liter, 0.5 cc. = 1.0 mg. ethyl iodide). This is made up according to Van Slyke's directions (7). (2) Potassium sulfocyanate solution (approximately 1 gm. per liter). (3) Powdered ferric ammonium sulfate. (4) Concentrated nitric acid (specific gravity 1.42).

Procedure.—250 cc. of air containing 2 mg. or less of ethyl iodide are collected in the sampling tube. 2 cc. of standard silver nitrate in nitric acid are pipetted into the test-tube and about 1 cc. of distilled water added.[6] One of the rubber connections of the sampling tube is wet with distilled water and the end of the test-tube engaged in it. The pinch-cock is opened and the test-tube thrust in until the glass end of the sampling tube overlaps it an inch or more. The tubes are now tilted to permit the standard to run in, then leveled, and rotated to wet the walls. A glass plug is substituted for the remaining pinch-cock and the apparatus put aside for 16 hours or more (we leave it overnight). In a few minutes the fluid becomes turbid and within an hour it is full of a flocculent yellow precipitate.

Next day the glass plug and its rubber connection are removed and the contents of the tubes emptied into an evaporating dish. The sampling tube and the attached test-tube are twice washed out with 5 cc. of distilled water. The test-tube is then withdrawn until it is no longer overlapped and the apparatus washed with 2 cc. more water to reach any standard which may have been caught between the overlapping parts. The washings are added to the evaporating dish which then contains about 14 cc. 2 cc. of con-

[5] For illustration see Henderson and Haggard, *Am. J. Physiol.*, 1925, lxxii, 236 (Fig. 6). Greased stop-cocks cannot be employed as grease absorbs ethyl iodide.

[6] If the vapor has been collected by water displacement, considerable water will remain in the bulb and the water added here should be correspondingly reduced.

centrated nitric acid are now added,[7] then about 0.3 gm. of powdered ferric alum, and the analysis is completed by titrating with the potassium sulfocyanate, according to the Whitehorn technique (8). The end-point[8] is the first definite russet color to last 15 seconds in spite of stirring. As the color change following each of the next 2 drops is marked we always confirm the position of the end-point by adding a drop or two more.

Calculation.

$$\frac{\text{Titer of unknown (in cc.).}}{\text{Titer of 1 cc. of standard } AgNO_3 \text{ (in cc.).}} = A$$

$$(\text{Standard } AgNO_3 \text{ added to tube (in cc.)} - A) \times 2 = \text{mg. } C_2H_5I$$

Remarks.—The reaction must go to completion in the time allowed. Its speed varies with the concentration of acid and silver nitrate present. The effect of various dilutions of the standard has been determined and the results are recorded in Table III. An initial concentration between approximately 50 and 90 per cent of concentrated nitric acid, and approximately 0.007 M and 0.012 M silver nitrate, is satisfactory. Above this range nitric acid is too unstable, the bulb fills with brown fumes, exposed rubber is attacked, and low results are often obtained. Below this range we have no confidence that the reaction will go to completion in 16 hours. We aim to dilute the standard by one-half its volume of water in the analyses. To maintain a suitable concentration of silver nitrate we add at least twice the amount necessary to react with the ethyl iodide. Room temperature is sufficient but

[7] The amount of acid added should be adjusted to the amount of standard employed to permit titration in 25 per cent HNO_3, as used by Whitehorn. In iodide methods this acidity is not essential to the titration in the presence of the precipitate (Volhard, cited from Treadwell, F. P., Analytical chemistry, translated by Hall, W. T., New York, 6th edition, 1924, 605).

[8] The KCNS is weaker than that employed by Whitehorn, but stronger than that used by Myers and Short (Myers, V. C., and Short, J. J., *J. Biol. Chem.*, 1920, xliv, 47). The volume of solution at the end-point is smaller than in the Whitehorn method, which partly offsets the effect of the weaker KCNS on the sharpness of the end-point. We have confirmed the position of the end-point by titrating standard KI solutions.

XUM

TABLE III.

Silver Nitrate Method.

Effect of Different Dilutions of Standard $AgNO_3$ in HNO_3.

Tubes remained at room temperature for 16 hours before titration unless otherwise noted.

Date.	Water added to 2 cc. standard.	Ethyl iodide added (weighed).	Ethyl iodide found.	Error.		Remarks.
	cc.	*mg.*	*mg.*	*mg.*	*per cent*	
Apr. 17	0	1.58	1.11	−0.47	−29.8	44 hours before titration. Tubes full of brown fumes. Rubber corroded. End-point ruined.
	0	2.19	1.80	−0.39	−17.8	
	0	0.22	0.17	−0.05	−22.7	
" 30	0.25	2.22	2.13	−0.09	−4.1	Very slight corrosion.
	0.25	0.91	0.92	+0.01	+1.1	No corrosion.
" 28	0.5	1.39	1.37	−0.02	−1.4	
	0.5	1.15	1.15	0	0	
" 21	1.0	1.49	1.50	+0.01	+0.7	
	1.0	1.46	1.38	−0.08	−5.5	
	1.0	1.38	1.39	+0.01	+0.7	
" 23	2.0	1.10	1.08	−0.02	−1.8	
	2.0	2.41	2.36	−0.05	−2.1	
" 26	4.0	0.87	0.81	−0.06	−6.9	Ppt. milky.
		1.25	1.11	−0.14	−11.2	AgI not flocculent.
Mar. 29	$AgNO_3$ without HNO_3*.	1.79	1.00	−0.79	−44.1	Ppt. milky.
	" "	3.45	1.93	−1.52	−44.1	" " 22 hrs.

* 1 cc. = 1 mg. of ethyl iodide. 5 cc. added for smaller, 7 cc. for larger amount of ethyl iodide.

it must be maintained. Under these conditions the reaction goes to completion within 7 hours (Table IV). To wait 16 hours allows ample margin of safety.

The concentrated acid should be kept away from the rubber. This is accomplished by wetting the inside of the rubber connec-

tion before sliding in the test-tube. This water occupies the space between the interlocking tubes and protects the rubber. At the other end a well fitting glass plug minimizes the contact between rubber and the interior. Acid must not be allowed to flow into this end. A slight contact between acid and black pure gum rubber connections does not interfere with the analysis. In spite of these precautions the rubber connections weaken and must be replaced every few weeks.

The apparatus and reagents must be free of soluble halogens. Traces of silver iodide remaining in the tubes in spite of washing do not interfere with subsequent analyses.

TABLE IV.

Silver Nitrate Method.

Speed of Reaction.

1 cc. H_2O + 2 cc. standard $AgNO_3$ in HNO_3 added to dry bulbs filled with equal amounts of ethyl iodide vapor. Temperature between 25–26° at all observations.

Time after start of reaction.	Ethyl iodide found.	Per cent of total.
hrs.	*mg.*	
1	0.78	55
3	1.13	80
5	1.28	91
7	1.40	99
24	1.41	100
48	1.41	100

As no other volatile halogens are likely to be encountered in physiological investigations the method may be regarded as determining ethyl iodide alone. While the results are not obtained until next day the method requires little more of the operator's time than the iodine pentoxide procedure.

Determination of Known Amounts of Ethyl Iodide Vapor.

Pure ethyl iodide[9] was drawn into small glass bulbs whose capillary outlet could be sealed without change of weight. The bulbs empty and loaded, were weighed on a Troemner balance advertised

[9] We are indebted to Dr. B. F. Freeman for the purification of the ethyl iodide used. It was practically colorless and boiled between 72° and 72.5°.

accurate to 0.05 mg., but certainly accurate to 0.03 mg. The swings were recorded but the weight was not calculated until after the analysis. A loaded bulb was inserted into a 250 cc. gas sampling tube together with a piece of glass rod, the ends of the tube were plugged, and the bulb demolished by shaking. After allow-

TABLE V.

Silver Nitrate Method.

Determinations of Weighed Amounts of Ethyl Iodide (Vaporized).

Date.	Weighed.	Found.	Error.	
			Absolute.	
Apr.	*mg.*	*mg.*	*mg.*	*per cent*
6	0.92	0.94	+0.02	+2.2
7	2.13	2.15	+0.02	+0.9
8	2.22	2.12	−0.10	−4.5
8	0.65	0.65	0	0
9	1.62	1.53	−0.09	−5.5
9	0.12	0.12	0	0
9	1.78	1.74	−0.04	−2.2
13	2.31	2.35	+0.04	+1.7
13	1.41	1.43	+0.02	+1.6
13	0.90	0.91	+0.01	+1.1
14	0	0.01	+0.01	
14	0	0.01	+0.01	
19	1.70	1.70	0	0
19	1.19	1.17	−0.02	−1.7
20	2.32	2.23	−0.09	−3.9
20	1.23	1.16	−0.07	−5.7
20	1.71	1.64	−0.07	−4.1
21	1.49	1.50	+0.01	+0.7
21	1.46	1.38	−0.08	−5.5
21	1.38	1.39	+0.01	+0.7

Mean difference	−0.02 mg.
Average deviation from mean difference	0.04 "
Maximum " " " "	−0.08 "

ing a few minutes for the ethyl iodide to volatilize, the standard was added and the analysis continued as described. The results of twenty consecutive determinations on amounts of ethyl iodide from 2.32 mg. to 0 are recorded in Table V, the maximum error being 5.7 per cent, the average error 2.3 per cent. It must be

remembered that there is a possible error of 0.06 mg. in the two weighings, also possibilities of error in loading the bulbs which would not occur in an ordinary analysis. Analyses of equivalent gas samples taken from the spirometer have consistently checked within 0.02 mg.[10]

Determination of Ethyl Iodide in Water.

The method described for vapor may be modified for the determination of ethyl iodide in halogen-free water. The sample is drawn into a grease-free glass syringe and is injected into a closed 250 cc. sampling tube. Sufficient standard is now added to give proper silver nitrate and nitric acid concentrations and the analysis continued as described for vapor.

Ethyl iodide slowly decomposes in water according to the equation (9):

$$C_2H_5I + H_2O = HI + C_2H_5OH$$

Care should be taken that appreciable amounts of hydrogen iodide have not accumulated in the water at the time when an analysis of its ethyl iodide content is desired, the method determining the total halogens.

Determination of Ethyl Iodide in Blood.

The ethyl iodide is driven off by boiling the blood in a vacuum, caught in a large tube, and determined by the addition of silver nitrate.

Apparatus.—This consists of three pieces: (1) A glass gas-collecting tube of 750 or 1000 cc. capacity. One of the end tubes is closed by a rubber connection and screw clamp; on the other is a joint ground to fit the volatilizing tube. Behind this joint a small bulb has been blown. (2) A volatilizing tube of about 65 cc. capacity. A bulb has been blown close to its joint. To the thick walled tube at its end a small needle valve is attached by a rubber connection. The volatilizing tube and the adjacent parts of the gas collecting tube are shown in Fig. 1. (3) A small test-tube, its end ground to fit the collecting tube after the volatilizing

[10] Four 250 cc. samples drawn from the spirometer = 1.30, 1.30, 1.30, 1.31 mg. Five other samples = 1.37, 1.36, 1.38, 1.36, 1.38 mg.

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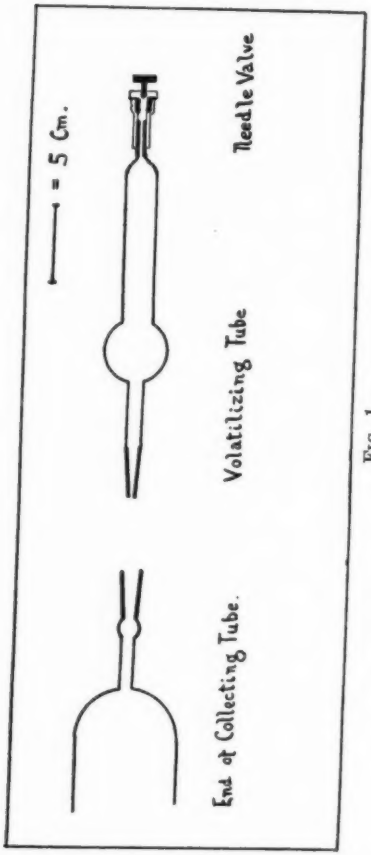

FIG. 1.

tube has been removed. (1) and (2) interlocked, the joint reinforced by a rubber connection, are supported with their long axis about 10° from the horizontal (needle valve down) so that they may be rotated about this axis.

Procedure.—The collecting tube and the volatilizing tube interlocked are exhausted to 45 mm. of mercury by a pump, and placed on their supports. The blood is collected in a glass grease-free syringe equipped with a long thin needle. The rubber connection of the needle valve is slid part way along the glass tube, bent down, and the needle thrust through the wall well into the tube. The negative pressure sucks the blood in. 0.5 cc. of Locke's solution containing a drop of caprylic alcohol, injected from another syringe, washes the last drops into the end of the volatilizing tube. The connection is then slid back to prevent leakage through the punctures.

The apparatus is now rotated on its long axis by one hand while the volatilizing tube and the adjacent parts of the collecting tube are being heated by playing a stream of hot water upon them. The blood, spread over a considerable surface by the rotation, is boiled until it no longer seeks the lowest point, most of its volatile contents condensing in the collecting tube. The needle valve is slowly opened and the gaseous contents of the volatilizing tube are washed into the collecting tube by the inrushing air. As soon as the hissing ceases the volatilizing tube is detached from the collecting tube, the rubber connection being pinched to prevent loss of gas until the test-tube containing 4 cc. of standard is inserted. After the collecting tube has cooled it is vented, the screw clamp replaced by a glass plug, and put aside for 48 hours.

The large tube and its attached test-tube are then emptied and washed with 5, 5, and 2 cc. of distilled water, each washing being allowed to drain completely into the evaporating dish before adding the next.[11] A final washing of 12 cc. is tested for the presence of silver nitrate, and this being negative, the titration is continued as described.

Remarks.—The fixation of ethyl iodide vapor occurs more slowly in the large tubes than in the smaller ones used previously.

[11] The increased sharpness of the end-point obtained by keeping the volume small amply compensates for the care and time required to wash out completely large bulbs with small amounts of water.

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4 cc. of standard provide suitable acid and silver nitrate concentrations in the presence of the water distilled from 2 to 5 cc. of blood. At room temperature the reaction goes to completion within 24 hours as shown in Table VI. We prefer to allow 48 hours to assure ample margin of safety.

If any blood is driven into the collecting tube the analysis must be abandoned. Little difficulty will be encountered in handling amounts of 3 cc. or less. We have often handled 5 cc. successfully. All parts of the volatilizing tube must be kept hot enough to prevent condensation.

TABLE VI.

Silver Nitrate Method.

Determination of Known Amounts of Ethyl Iodide Added to Blood.

Titrated 24 hours after the addition of the standard.

Date.	Amount of blood.	Ethyl iodide added (by analysis).	Ethyl iodide found.	Error.	
				Absolute.	
	cc.	*mg.*	*mg.*	*mg.*	*per cent*
June 22	0.5	0.95	0.89	−0.06	−6.3
" 24	3	1.56	1.57	+0.01	+0.6
" 25	2	1.51	1.50	−0.01	−0.7
" 26	2	1.34	1.28	−0.06	−4.5
" 28	2.5	0.58	0.56	−0.02	−3.4
" 29	3	0.14	0.14	0	0
" 30	3	0	0.02	+0.02	
July 2	3	1.01	0.95	−0.06	−6.0
" 3	5	0.74	0.75	+0.01	+1.4

Mean difference	−0.02 mg.
Average deviation from mean difference	0.03 "
Maximum " " " "	−0.04 "

The hurried detachment of the tubes sometimes results in breaking the ground glass joint. This can be minimized by thoroughly wetting the joint and its connection before interlocking. Thick glass should be used in making the joint.

The decomposition products of ethyl iodide, HI and C_2H_5OH, do not interfere with this method, the former being converted into non-volatile salts in the blood, the latter not reacting with silver nitrate. This is an obvious advantage over the iodine pentoxide procedure.

Determination of Known Amounts of Ethyl Iodide in Blood.

Between 0.01 and 0.1 cc. of ethyl iodide was added to a tonometer containing about 10 cc. of distilled water. The tonometer was rotated for 5 minutes. About 2 cc. of the water were withdrawn into a glass grease-free syringe calibrated both for capacity and delivery. About one-half its content was injected into a closed 250 cc. sampling tube and its ethyl iodide content determined. Sufficient 3.6 per cent NaCl was then drawn into the syringe to make it contain 0.9 per cent NaCl. Blood, kept fresh by oxalate or fluoride, was then drawn into the syringe and rotated

TABLE VII.

Iodine Pentoxide Method.

Loss of Ethyl Iodide from Wet Tubes Closed by Rubber Tubing and Pinch-Cocks.

Loss figured from average of three early analyses.

Tube No.	Time between sampling and analysis.	Titration thiosulfate.	Loss.
	hrs. min.	*cc.*	*per cent*
1	0 16	14.95	
5	0 22	15.01	
2	0 28	14.98	
4	1 29	13.88	7.3
11	2 08	14.02	6.4
13	4 29	13.15	12.2
7	6 05	13.30	11.2
17	6 10	13.55	9.6

for 5 minutes. The contents of the syringe were then analyzed as described. The amount of ethyl iodide added to the blood was calculated from the analysis of the water and compared with that recovered from the blood. Table VI shows the results of nine consecutive determinations. The maximum error is 6.3 per cent, the average error 2.9 per cent.

Methods of Collection and Preservation of Ethyl Iodide Vapor.

Samples may be collected without loss by allowing water to run rapidly out of sampling tubes of 760 cc. capacity or less. We leave at least 50 cc. of air between the water surface and the

TABLE VIII.

Data on Methods of Collection and Preservation of Ethyl Iodide in Air.

Date and purpose.	Time.	Capacity of sampling tube.	Concentration of ethyl iodide found.	Method of collection and remarks.
1926		*cc.*	*mg. per 250 cc.*	
May 10	12.00	250	1.23	Tube dry. 20 liters run through before sampling.
Collection by rapid water displacement.	12.10	250	1.24	Drawn in by distilled water flowing rapidly out (15 sec.).
	12.20	250	1.21	Dry.
	12.22	250	1.22	Drawn in by water (15 sec.).
May 11	10.00	250	1.12	Drawn in by water (15 sec.).
Same in large tubes.	10.05	760	1.14	" " " " (30 ").
	10.10	250	1.14	" " " " (15 ").
Sept. 16	2.35	250	1.72	Drawn in by distilled water (15 sec.).
Slow displacement with acidified water.	2.40–2.46	250	1.73	Acidified water (0.5 per cent HNO_3) flowing out in 6 min.
	2.49–2.59	250	1.69	" " flowing out in 10 min.
	3.05	250	1.68	Drawn in by distilled water (15 sec.).
May 6	2.00	250	0.61	Dry.
Error in slow displacement in a large tube.	2.10	760	0.49	Drawn in by distilled water flowing out in 11 min. 250 cc. of air between water surface and air analyzed.
Sept. 15	12.35	250	1.46	Spirometer concentration (rapid water displacement) with which rubber bag was washed out three times and then filled.
Rapid loss of ethyl iodide from a rubber bag.	12.47	250	0.94	Concentration recovered from bag 5 min. after filling (rapid water displacement).
	12.52	250	0.71	Concentration recovered 10 min. after filling.
	2.12	250	1.45	Spirometer concentration (dry).

	2.12	250	1.45	Spirometer concentration (dry).
Sept. 15	12.35	250	1.46	Spirometer concentration.
Slight loss of ethyl iodide on passage through rubber tubing.	12.42			Rubber tube 6 meters long, 0.3 cm. internal diameter, washed out by 11 liters from spirometer.
	1.15–1.40	250	1.39	Sample collected beyond tube. Flow through tube = 0.5 liter per min.
	1.40–2.08	250	1.38	Second sample collected beyond tube.
	2.12	250	1.45	Spirometer concentration.
May 10	12.00	250	1.22	Spirometer concentration with which brass bag was washed out three times and then filled.
Slow loss of ethyl iodide from brass bag.	12.05	250	1.22	Concentration recovered from bag 5 min. after filling.
		250	1.15	" " 10 min. after filling.
		250	1.12	" " 15 " " "
Apr. 12			1.42	Theoretical concentration when spirometer is loaded with 1 cc. pure ethyl iodide (fan on).
Loss in filling spirometer.			1.30	Concentration found by analysis 5 min. after filling (average of four determinations).
May 10			1.36	Concentration in spirometer (average of five determinations).
Loss in standing in spirometer.			1.13	" found 24 hrs. later (fan off). (Average of two determinations.)

air analyzed. If the water runs out slowly there is danger of loss in spite of this precaution. 0.5 per cent nitric acid in water is the best fluid for slow displacement that we have found. If mercury is employed, the gas must be transferred as enough remains on the walls to interfere with the titration.

The error of preserving ethyl iodide in wet bulbs closed by pinch-cocks is large when they are analyzed by the iodine pentoxide method (Table VII). By adding standard promptly after securing the sample and substituting glass plugs for pinch-cocks the silver nitrate method avoids this error.

When a known amount of ethyl iodide is volatilized in the spirometer,[12] the theoretical concentration cannot be recovered. If the spirometer has been standing free of ethyl iodide, the loss will amount to about 10 per cent. If it has contained ethyl iodide, dilution by air yields a higher concentration than anticipated. These changes are probably due to loss of ethyl iodide to the water or gain from it. Adsorption may also be a factor, but ethyl iodide does not seem to be easily adsorbed, passing through tubes packed with glass wool without difficulty. After an initial loss the concentration in the spirometer falls slowly probably due to the slow solution and destruction of ethyl iodide in the water.

The ethyl iodide concentration in air in a rubber bag falls with surprising rapidity. There is a small loss when it flows through rubber tubing. We have made a fairly satisfactory rebreathing bag out of two thin sheets of spring brass, their edges being soldered together and a tube inserted for the mouthpiece. The concentration within it slowly diminishes. This error is not detectable for 5 minutes, which gives ample time to transfer the gas to a sampling tube.

Our data on the collection and preservation of ethyl iodide are recorded in Table VIII. We collect the vapor in a dry tube or by rapid displacement of distilled water, adding standard immediately after collecting. Our room, not reached by the sun and adjacent to a large laboratory, is not subject to rapid variations of temperature.

A glass syringe rendered grease-free, the plunger kept tight by wetting with distilled water, seems the most satisfactory

[12] The spirometer was of 300 liters capacity, its gas chamber painted with red lead.

method of handling ethyl iodide in water or blood. It is calibrated for capacity and delivery, and fluid can be measured in it with accuracy.[13] A correction is made for the small dead space.

TABLE IX.

Coefficient of Distribution of Ethyl Iodide in Air and Water at 37°C.

Date.	Duration of equilibration.	Size of sample.	Ethyl iodide found by analysis.	Ethyl iodide concentration.	Coefficient.
	min.	*cc.*	*mg.*	*mg. per 100 cc.*	
May 19	Before.	Gas. 250.0	7.29	2.92	
	3	Water. 1.9	0.13	6.84	2.4
	8	" 2.0	0.17	8.5	2.9
	14	" 2.0	0.15	7.5	2.6
	24	" 2.0	0.16	8.0	2.8
	44	" 2.0	0.18	9.0	3.1
	After.	Gas. 103.5	2.97	2.87	
May 24(a)	Before.	Gas. 250.0	8.31	3.32	
	3	Water. 2.0	0.15	7.5	2.3
	6	" 2.0	0.17	8.5	2.6
	13	" 2.0	0.16	8.0	2.4
May 24(b)	5	Water. 1.95	0.17	8.7	2.7
	10	" 2.0	0.19	9.5	3.0
	18	" 2.0	0.18	9.0	2.8
	44	" 2.0	0.18	9.0	2.8
	After.	Gas. 103.5	3.26	3.15	

100 minutes between gas samples on May 24.

Average of eight coefficients determined between 4 and 25 minutes = 2.7.

Coefficient of Distribution of Ethyl Iodide in Air and Water.

A preliminary experiment was performed to determine the order of magnitude of the destruction of ethyl iodide in water. Air containing ethyl iodide was allowed to flow through a tonometer containing phenolsulfonephthalein in distilled water. A slow change of color was observed. Calculation of the acid formed was necessarily inexact, but indicated that the destruction of ethyl

[13] The syringe loaded to the 2 cc. mark with samples of distilled water delivered 2.018, 2.031, 2.024, and 2.022 gm.

iodide was so slow that it would not be chemically detectable in a short experiment. Later analytical results supported this conclusion.

Ethyl iodide vapor was led from the spirometer through a glass spiral and a sampling tube immersed in a water bath at 37° (± 0.5°). After 10 liters had passed through, the sampling tube was removed for analysis and a tonometer containing distilled water inserted in its place. The tonometer was constantly rotated from side to side while the stream of vapor flowed through it at 2 liters per minute. At intervals the flow was stopped and samples of water removed for analysis. A second gas sample terminated the experiment.

The results of three experiments, recorded in Table IX, agree well within the chemical error involved. Omitting samples taken before 4 minutes of equilibration as possibly unsaturated and those taken after 30 minutes as possibly having accumulated detectable amounts of decomposition products, the average of seven analyses gives a coefficient of 2.7.

Coefficient of Distribution of Ethyl Iodide in Air and Blood in Vitro.

Human blood, kept fluid by oxalate or fluoride was saturated by the technique employed for water. The results (Table X) fall into two groups, anemic and normal. Plasma has a far lower coefficient than whole blood from the same individual, and anemic blood has a correspondingly lower coefficient than normal. Five normal bloods yield figures within the probable error of the determination, and their average 7.6 we believe to be the correct coefficient.

This coefficient for blood is far higher than that for water. A similar relationship was found for ethyl chloride by Embley (10), the coefficient being 2.5 for water at 21°C. and exceeding 5.0 by an undetermined amount for blood at 38°C.

It should be pointed out that the more severe anemia has the higher coefficient. This is perhaps within the chemical error involved, but it seems probable that other factors than the red cell count and hemoglobin may influence the coefficient. The assumption of the same coefficient for all persons is not justified.

TABLE X.

Coefficient of Distribution of Ethyl Iodide in Air and Blood at 37°C.

Date.	Source of blood.	Amount in tonometer.	Time equilibrated.	Observed size of samples.	Ethyl iodide found by analysis.	Ethyl iodide in blood.	Ethyl iodide in air before and after equilibration.	Coefficient.
July		*cc.*	*min.*	*cc.*	*mg.*	*mg. per 100 cc.*	*mg. per 100 cc.*	
7	Patient H.						4.05	
	(blood	10	10	1.95	0.46	23.7		
	normal).		25	2.0	0.56	28.1		7.1
			40	1.95	0.58	29.9		
8	Patient D.						1.95	
	(blood	6	13	2.0	0.31	15.6		
	normal).		32	2.0	0.32	16.1		8.2
							1.93	
14	Patient F.						2.90	
	(blood	10	65	2.15	0.46	21.5		7.5
	normal).						2.87	
19	Subject S.						3.18	
	(blood	8	42	2.4	0.59	24.7		7.8
	normal).						3.18	
22	Subject B.						3.67	
	(blood	10	30	3.0	0.79	26.4		
	normal).		45	2.2	0.65	29.6		7.6
							3.68	
24	Subject B.						3.51	
	(plasma).	8	28	2.2	0.35	16.0		
			50	3.0	0.54	18.0		4.9
							3.46	
12	Patient G.						3.00	
	Hb = 48 per cent	16	54	3.6	0.53	14.7		
	R.b.c. = 3,990,000		73	4.8	0.78	16.3		5.2
							2.96	
26	Patient M.						3.68	
	Hb = 22	9	72	2.2	0.49	22.4		
	R.b.c. = 1,070,000		97	1.6	0.40	25.1		6.5
							3.59	

In calculating the concentration the observed size of samples was corrected slightly for the calibration and dead space of the syringe.

The coefficients are calculated from the average of the gas concentrations and the last two blood analyses.

Average of five determinations on normals = 7.6.

Rate of Destruction of Ethyl Iodide in Blood and Its Elimination by the Lungs.

We have obtained evidence that the destruction of ethyl iodide in blood is a slow process. In the determination of known amounts of ethyl iodide in blood, the blood and ethyl iodide were equilibrated for 5 minutes before analysis without detectable

TABLE XI.

Silver Nitrate Method.

Ethyl Iodide in Alveolar and Expired Air after Breathing It.

Date.	During inhalation of ethyl iodide.			After inhalation of ethyl iodide.				
	Inspired air.	Expired air.	Krogh-Henderson alveolar air.	Duration of air breathing before rebreathing.	Duration of rebreathing.	Concentration in rebreathed air.	Expired air.	Per cent of alveolar concentration in rebreathed air.
	mg. per 250 cc.	*mg. per 250 cc.*	*mg. per 250 cc.*		*sec.*	*mg. per 250 cc.*	*mg. per 250 cc.*	
Oct. 5	1.47	0.73	0.36	0	35	0.12		33
				4 min.	40	0.06		16
May 14	1.69	0.81	0.42	0	25	0.14		33
" 13	1.44	0.78	0.32	5 sec.	35	0.09		28
Oct. 4	1.48	0.70	0.36	10 "	15	0.08		22
" 8	1.76	0.81	0.38	20 "	30	0.09		24
				3 min.	30	0.06		16
				6 "	30	0.02		5
Sept. 17	1.45	0.73		5 "			0.05	
				10 "			0.03	

Subjects lying down, rested at least ½ hour before all experiments. Subjects fasted on Oct. 4, 5, and 8. 1000 cc. of air in brass bag used for rebreathing. 500 and 750 cc. tubes used to take samples of rebreathed air. Expired air samples taken from 4 liter mixing bottle stirred by a fan. Subjects breathed ethyl iodide for 10 minutes.

loss. Two 5 cc. samples of dog's blood, kept fluid by oxalate, which originally contained 1.37 mg. of ethyl iodide, were preserved in syringes for 21 hours at 37°C. Analysis then showed 1.05 and 1.01 mg., a loss of only 23 and 26 per cent.

Before devising a technique for the determination of ethyl iodide in blood we attempted to determine the coefficient of dis-

tribution by analyzing vapor before and after its equilibration with blood, the amount dissolved being determined by difference. The difficulty of measuring gas volumes made the experiments very crude but the average of three determinations gave a coefficient of 8.2 after from 6 to 12 minutes of equilibration and of 10.0 after from 20 to 30 minutes. Experiments on plasma also showed a slowly rising coefficient. In confirmation of other workers (11) we have detected ethyl iodide in the alveolar and expired air after breathing it. The alveolar air samples were obtained by the Higgins-Plesch technique (12) beginning with 1000 cc. in the brass bag and rebreathing for about 25 seconds. The expired air samples were drawn from a 4.0 liter mixing bulb stirred by a fan. Large sampling tubes (up to 760 cc.) were used to minimize the chemical error. Typical experiments are recorded in Table XI. The results show that the excretion of ethyl iodide takes place slowly. Its characteristic odor can be detected on the breath for hours. It is not completely destroyed in the body. It must be carried to the lungs by the venous blood after and consequently during the inhalation of ethyl iodide.

The return of ethyl iodide to the lungs during a determination constitutes a fundamental error in the ethyl iodide blood flow method. Theoretically the size of this error could be determined if it were possible to get rebreathed air into equilibrium with the mixed venous blood within one circulation time after ceasing to breathe ethyl iodide. We have tried the procedure of rebreathing for about 30 seconds after turning off the ethyl iodide. Two chief errors may be involved. There is evidence that in 30 seconds part of the blood has returned to the lungs a second time (13). The persistence of the elimination of ethyl iodide after ceasing to inhale it indicates that the tension in the tissues supplies the venous blood with ethyl iodide after the arterial concentration has fallen. Therefore, only a small error would result if some part of the blood returned a second time within the period of rebreathing. Of greater importance is the possibility that equilibrium may not be attained in 30 seconds. After breathing ethyl iodide the lungs are full of and their tissues saturated with a higher concentration of ethyl iodide than in the venous blood, and if equilibrium were not attained the results would be too high. We have in some experiments taken deep breaths from fresh air before rebreathing. This

should result in an initial concentration of ethyl iodide in the rebreathed air below that in equilibrium with mixed venous blood and failure to reach equilibrium would give a low result. Table XI shows that the results of these types of experiment (May 14 and 13, October 5 and 8) approach each other, their mean being in the neighborhood of 29 per cent of the previous Krogh-Henderson alveolar concentration. This procedure is similar in principle to the "straddling" method of obtaining samples of gases in equilibrium with mixed venous blood described by Christiansen, Douglas, and Haldane (14) and depends on the assumption that ethyl iodide is not given off by or absorbed into the lung fluids and tissues in sufficient quantities to influence the result. We do not regard the exact amount of ethyl iodide in the venous blood as established.

DISCUSSION.

By means of a chemical technique which we believe superior to that previously available we find the coefficient of distribution of ethyl iodide in air and normal blood to be 7.6. Results of this order of magnitude were obtained by previous investigators (15), but were disregarded because of the belief that ethyl iodide was rapidly destroyed in blood, the iodine pentoxide method determining both ethyl iodide and its decomposition products. In the experiments in which a coefficient of 2 was obtained the surface of contact and the duration of exposure were extremely small in comparison with standard methods for the equilibration of blood with oxygen or carbon dioxide (16). Therefore we attribute the low results to failure to saturate with ethyl iodide.

We submit evidence that ethyl iodide is not rapidly and completely destroyed in blood or in the body, that it is eliminated by the lungs and so must occur in the venous blood during a blood flow determination, causing an error. The failure of others to find equivalent amounts of ethyl iodide in the alveolar and expired air after inhaling it (17) may well have been due to the rapid disappearance of ethyl iodide from the rubber bags used to collect the samples. The conception of a rapid destruction of ethyl iodide in blood *in vitro* (18) is due to underestimating its solubility.

In the presence of these errors how did we and others (19, 20)

obtain results resembling blood flow with the ethyl iodide method? Apparently because the two chief errors tend to cancel each other. It should be pointed out that by assuming that the sample obtained by rebreathing for 25 seconds after inhaling ethyl iodide approaches equilibrium with the mixed venous blood, and by using a coefficient of 7.6, figures for blood flow are obtained lower than those calculated without these corrections, but in good agreement with other blood flow methods.[14]

Evidence is accumulating that for subjects at rest samples in equilibrium with arterial blood are best obtained by the Haldane technique (21, 22). To correct for this would raise the blood flow figures somewhat.

We believe that it may be possible to measure the circulation by means of ethyl iodide but the procedure cannot be as simple as that described by Henderson and Haggard. Experiments on this subject are being continued.

SUMMARY.

1. A method is described for the determination of ethyl iodide in air by means of its reaction with silver nitrate, the average accuracy being 2.3 per cent for amounts from 2.35 to 0.12 mg. Adaptations of this method for the determination of similar amounts of ethyl iodide in water and blood are given. Satisfactory methods for the collection of samples of ethyl iodide in air and fluids are described.

[14] Experiment 1. Subject lying at rest. Pulse 70. Respiration 110.5 liters in 15 minutes, inspired air = 1.44 mg. per 250 cc., expired air = 0.78 mg. Krogh-Henderson alveolar air 0.32 mg., Plesch 0.09 mg. Blood flow figured according to Henderson and Haggard 7.6 liters per minute. Corrected for ethyl iodide in venous blood and using coefficient of 7.6 (= 7.1 to correct for sampling at room temperature) blood flow equals 3.0 liters per minute. Corrected to Haldane alveolar air, blood flow = approximately 3.1 liters per minute. Experiment 2. Subject lying at rest. Respiration 117 liters in 15 minutes, pulse 72, inspired air = 1.69 and 1.70 mg. per 250 cc., expired air 0.79 and 0.84 mg., alveolar 0.42 mg. Plesch 0.14 mg. Calculated according to Henderson and Haggard blood flow = 8.2 liters per minute. Corrected for ethyl iodide in venous blood and using coefficient of 7.6 blood flow = 3.5 liters per minute. Corrected to Haldane alveolar air blood flow = approximately 3.6 liters per minute.

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2. Evidence is submitted that the iodine pentoxide method for the analysis of ethyl iodide is not reliable in the form described.

3. Using the new technique, the coefficient of distribution of ethyl iodide in air and water has been determined to be 2.7; in air and normal blood *in vitro* to be 7.6 instead of 2.0 as previously believed. Plasma and anemic blood have a lower coefficient than normal blood.

4. Evidence is submitted that the destruction of ethyl iodide in water and blood *in vitro* is a slow rather than a rapid process; that ethyl iodide is not completely destroyed in the body, but is eliminated by the breath and so must occur in the venous blood during its inhalation.

5. That the ethyl iodide method for the determination of blood flow gives figures consistent with other methods appears due to the fact that the errors tend to cancel each other. In its present form the results cannot be accepted.

We are indebted to Professor A. N. Richards for the suggestion that led to this investigation and for much advice during the work. We are also indebted to Professors Henderson and Haggard for personally instructing us in the use of the ethyl iodide blood flow method and for supplying us with iodine pentoxide and other necessary materials.

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CONCERNING PSEUDOMORPHINE.*

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Pseudomorphine (oxydimorphine, dehydromorphine) is not well known. It has been studied only in a desultory way and over a long period of years. Even its existence as a chemical entity is sometimes doubted. Work in this laboratory has led to the preparation and study of this base, which exhibits some interesting and unrecorded peculiarities. The present paper aims to present a general and largely qualitative description of this substance.

Pseudomorphine was first described in 1835 by Pelletier (1), who made known several of its characteristic properties and noted its pharmacological inertness. Realizing its close relationship to morphine, Pelletier named the substance pseudomorphine, the designation used in this article. It was recognized as an oxidation product of morphine by Hesse (2), and the now accepted formula was first proposed by Polstorff (3), reasoning from a series of syntheses in the morphine derivatives. This was later confirmed through the molecular weight determinations of Bertrand and Meyer (4).

Pseudomorphine is regarded as 2 morphine molecules, each lacking 1 hydrogen and linked through the carbon. As shown by Hesse's acetylation of the base (5) the four hydroxyl groups are intact, yielding a tetraacetyl derivative, according to Vongerichten (6). This author was able to prepare a mono-, but not a dimethylpseudomorphine, and concluded that the phenolic properties of 1 hydroxyl were modified. The identification of pseudomorphine, and its differentiation from morphine, have rested almost entirely on color reactions, many of the best being described by Marmé (7) and Hesse (8). The insolubility of the base itself, and of its sulfate, a striking property though sometimes exaggerated, has been used, but rarely.

Theoretically, pseudomorphine should be the first oxidation product of morphine. As a matter of fact, its presence has been reported at times

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in almost every reaction where morphine is oxidized, particularly in oxidations by silver and copper salts (5, 9, 10); by permanganate (11); by air in the presence of alkali (12); as produced in the drying of opium; by the action of a tyrosinase-like ferment on morphine (13, 14); and as a degradation product of morphine in the animal body (15). While some of these observations, notably the last, have been disputed (16), the importance of pseudomorphine in studying the oxidation of morphine is undoubted.

Preparation.

Morphine may be conveniently oxidized by potassium ferricyanide (Polstorff (3)); or by hydrogen peroxide in the presence of potassium cuprous cyanide, which acts as a catalyst (Denigès (17)). The latter method was used. By adjusting the acidity of the solution to between pH 6.5 and 6.8, the yields can be somewhat increased, though a yield of 25 per cent is a good one.

The crude base is contaminated with morphine, cyanides, adsorbed salts, and a number of other morphine oxidation products. It must be carefully purified, and instead of crystallizing from ammonia (9), a quite different method was worked out: the resulting precipitate of crude base is redissolved in HCl (proper care being taken for the presence of HCN during the operation), then reprecipitated by ammonia at a pH of 6.5. Phenol red is a convenient indicator for testing the reaction. This is repeated, and no attempt is made to wash the second precipitate. Most of the morphine and all of the cyanide are removed in this way. The hydrochloride is again prepared and salted out as such by adding between 5 to 10 volumes per cent of concentrated HCl. Morphine hydrochloride is soluble under these conditions, its maximum insolubility occurring at about 5 volumes per cent. The hydrochloride is now quite free from morphine, but as an additional measure is recrystallized from a large volume of water, in which it is much more insoluble than the corresponding morphine salt. It is then collected and when dry boiled up with methyl alcohol containing a little HCl, filtered off, and again dried. All other oxidation products of morphine are thus removed, and the resulting heavy white powder is pseudomorphine hydrochloride.[1]

[1] Hesse (8) reported a basic hydrochloride of pseudomorphine, which it is believed this method of purification eliminates. The preparation on analysis was shown to contain 2 equivalents of HCl per equivalent of pseudomorphine.

Identification.

The compound was identified by its insoluble sulfate; by the composition of the silicotungstate, which follows the general rule of Bertrand (18) (when pseudomorphine is regarded as dibasic); and by the series of reactions proposed by Marmé (7) particularly to distinguish this base from morphine and codeine. Confirmation of the reported optical activity was not obtained, but this will be described at length in a later paper.

General Reactions.

The free base is easily prepared by neutralizing the salts with alkali. The precipitated base, however, becomes an amorphous mass on washing, so fine that it cannot be centrifuged properly, and so sticky that it cannot be filtered. It is readily coagulated by traces of electrolytes and adsorbs them.

Pseudomorphine is a weaker base than morphine, and a stronger acid. The isoelectric point has not yet been properly determined, but appears to be close to pH 8.0, with, however, a pretty wide isoelectric range, which makes possible its separation from morphine by precipitation in slightly acid solutions.

While the insolubility of the base was so striking a characteristic as to receive early comment (2), the reported solubilities of both the base and its salts show many contradictions. Observation of the preparations in this laboratory have shown the following.

Pseudomorphine is soluble in aqueous and (contrary to general belief) also in alcoholic ammonia, though more readily in the former. It is not precipitated by excess of ammonia. The results from any of the methods of separation from morphine depending upon these solubilities with ammonia are dubious. The base is soluble in most acids and in the caustic alkalies; sodium carbonate solution dissolves it only to a limited extent. The liquid hydrocarbons, the lower aliphatic ethers and esters, the lower alkyl halides, and water have apparently no solvent action on the dried and powdered base. Traces dissolve in hot butyl, amyl, or heptyl alcohol, and a small but measurable solubility occurs with the lower alcohols. The extraction of pseudomorphine by amyl or butyl alcohol in a funnel is practically

impossible, but in an efficient continuous extractor it may be partially removed by persistent extraction. This is an important consideration in regard to the usual methods of alkaloid isolation from biologic material, and it is doubtful whether these methods would even reveal the presence of pseudomorphine, except in unusually favorable cases. Certain cyclic liquids are good solvents for pseudomorphine. Among these are pyridine and aniline, perhaps because of their basic properties. Guiacol and the cresols also dissolve it, better on heating. The best solvents found in this laboratory are wood creosote, benzyl alcohol, and pyridine. A large number of mixtures were tried in hope of finding some suitable combination of solvents, but without success. This property of insolubility is continued in the various salts, all of which are comparatively less soluble than the corresponding morphine derivatives. The hydrochloride is a white poorly crystallized substance which analyses showed to be anhydrous when dried *in vacuo* or at 115°. The solubility in water was found to be about 1 part in 125, and this is rapidly decreased by the common ion effect of chlorides. Strong hydrochloric acid dissolves it, however. The aqueous solution hydrolyzes on dilution, slowly precipitating the free base. It is impossible, therefore, to prepare an acid or aqueous solution, except within a narrow range of concentrations.

The sulfate resembles the hydrochloride, except that it is less soluble. In methyl and in ethyl alcohol, the hydrochloride is practically insoluble, and the sulfate quantitatively so. This is a characteristic difference from morphine. The salts most soluble in water are the acetate, benzoate, and salicylate, and in alcohol particularly the last two. The picrate is fairly water-soluble, the picrolonate less so, while difficultly soluble compounds (resembling those with morphine) are formed with perchloric acid, lead chloride, and platinum chloride. Trichloroacetic acid precipitates an insoluble salt, which is not redissolved easily by alcohol or acetic acid (distinction from morphine).

The silicotungstate is thrown out of acid solutions as a finely divided, gelatinous, nearly white precipitate, which may be coagulated by electrolytes. When dried at 120° it differs from the corresponding morphine salt in color, and in containing no water of crystallization. It also differs in being much more insoluble in dilute acids and alcohol, and can even be completely

precipitated from hot solutions. The pseudomorphine salt can be distinguished from the corresponding precipitates of morphine, its other oxidation products, and of proteoses, peptones, etc., by its behavior in nearly neutral buffer solutions. While these other silicotungstates are easily soluble in phosphate buffers of pH 6.5, pseudomorphine silicotungstate is insoluble at pH = 8.0. This gives a valuable method of separating pseudomorphine from these similarly reacting substances. By adjusting the reaction of an alkaline solution of the mixed silicotungstates with KH_2PO_4 until a pH of 7.2 to 7.5 is reached (slightly alkaline to litmus paper), pseudomorphine is completely removed as a mixture of the silicotungstate and the free base.

Some of the reactions just discussed serve admirably as tests for pseudomorphine. The insolubility of the sulfate is useful, and also that of the base itself, combined with its peculiar appearance on first separating out of solution. This was described by Pelletier (1) as a mother of pearl luster and is easily recognized after a little experience. The isoelectric point of the base is such that the hydrolysis produced in nearly neutral solutions of its salts by such substances as potassium phenolsulfonate, potassium fluoride, and the like, is sufficient to cause this characteristic precipitation from even fairly dilute solutions. The presence of pseudomorphine in morphine may be recognized by adding such substances to the solution, or by using an excess of M/5 phosphate buffer of pH 6.5. Morphine will remain in solution; pseudomorphine is precipitated immediately.

The statement that pseudomorphine exerts no narcotic effect was confirmed by injecting a 5 kilo dog under the skin with 0.100 gm. of the hydrochloride. For an hour and a half the dog showed no unusual behavior and remained apparently normal for some days thereafter.

Pseudomorphine decomposes on either oxidation or reduction. In alkaline solutions, when shaken with air the pseudomorphine is gradually replaced by more highly oxidized bodies. It is therefore doubtful if much pseudomorphine can be obtained by the action of silver or gold salts on morphine. An ammoniacal solution of silver oxide was allowed to react with morphine for 15 days. During this time all of the morphine had disappeared but at no time during the process could the presence of more than a trace of pseudomorphine be demonstrated. A solution of

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morphine in caustic potash containing a trace of copper was shaken in the air. Rapid absorption of oxygen occurred, and the morphine soon disappeared. Again, however, only small traces of pseudomorphine were found in the solution at any given time.

On treating pseudomorphine with zinc dust and alkali the concentration diminishes, and bodies resembling morphine in precipitation with silicotungstic acid remain. The nature of these products of reduction has been left undetermined, awaiting a more generous supply of material, but that they are not morphine seems evident from the work of Donath (19) with similar reducing agents.

SUMMARY.

The preparation of pseudomorphine and a method of purification are described, the latter being based upon a rather wide survey of its chemical properties. The most striking properties are the insolubility of the base and many of its salts in organic solvents as well as in water, and the behavior of the silicotungstate in buffer solutions. Because of its insolubility, good solvents for the base are useful, and three have been found: pyridine, guiacol, and benzyl alcohol. A number of tests distinguishing pseudomorphine from morphine are described, applicable to mixtures of the two.

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THE SEPARATION AND ESTIMATION OF MORPHINE, PSEUDOMORPHINE, AND RELATED SUBSTANCES.*

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This paper aims to present a number of analytical methods, worked out in this laboratory, for studying systems in which morphine is gradually oxidizing. In order to approach a quantitative study it is necessary to possess a scheme of analysis for such rapidly changing systems whereby not only the residual morphine may be estimated, but also some information may be obtained concerning the amount of morphine-derived substances existing at various oxidation levels. The problem of devising such a scheme differs from the usual one of isolating and estimating morphine itself, not only by its object, but also by taking into account the properties of several other substances which must be estimated in the presence of each other.

When a solution of morphine undergoes gentle oxidation, some of the substances formed resemble morphine in many chemical properties. In particular they are capable of further oxidation, hence are reducing agents; their behavior with many reagents of alkaloid chemistry is like that of morphine; they are precipitated by the complex acids of tungsten and molybdenum; and they are comparatively insoluble in solutions near the neutral point.

The first of these oxidation products is theoretically pseudomorphine, formed by the removal of 1 equivalent of hydrogen per morphine. The occurrence of this substance is a possibility in any morphine oxidation, and its presence has been reported in a great number of cases, such as oxidation by gold or silver salts, by oxygen in alkaline solutions, by hydrogen peroxide, by tyro-

* This work was made possible by a grant from the Committee on Drug Addictions, New York City.

sinase-like ferments, and by many other means. Because of the probable formation of pseudomorphine at the very outset of morphine oxidation, its consideration in these methods was regarded as important, and the properties of pseudomorphine were first studied and have been given in a previous paper.

Besides pseudomorphine, a large number of other substances formed during the early course of the oxidation, are precipitated by phosphotungstic and silicotungstic acids, and apparently nearly as completely as morphine itself. If then silicotungstic acid be added to partially oxidized morphine in solution under proper conditions, it will precipitate pseudomorphine, morphine, and a heterogenous group of substances resembling morphine and pseudomorphine in many ways, but containing less hydrogen. To distinguish these substances from pseudomorphine and to classify them (for convenience) as a group, we shall call them "higher oxidation products of morphine," without regard to the fact that such a group is comprised of many substances probably of widely differing structure, some of which are still active reducing agents.

The quantity of these substances is, however, a measure of the amount of morphine oxidized beyond the pseudomorphine stage, and yet not far enough to loose all chemical resemblance to the parent alkaloid. Precipitability by silicotungstic acid becomes for the time being the criterion of this group, but it is not entirely an artificial one, because a very large proportion of the complex bodies formed on oxidation is precipitated in this way. This procedure has the advantage of almost instantaneously removing a large part of the morphine-derived substances from the further effect of the oxidant. It allows a mixture of continuously changing composition, in which the constituents sought are unstable, to be fixed at a given time and separated afterward.

The separation has been worked out to allow the determination of morphine-like substances at three levels of oxidation; namely, residual morphine, pseudomorphine, and the empirical class of higher oxidation products. From such analytical data, and in systems originally containing known amounts of morphine and oxidant, five variables may be evaluated as aids in following the reaction.

From the mixture of silicotungstates, pseudomorphine is sepa-

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rated first, and its removal allows an accurate determination of morphine, which is not possible, at least by any of the commonly described methods, in the presence of pseudomorphine. The morphine is next removed from the higher oxidation products and may be determined by any of the numerous accepted procedures. The precipitation method described is selected because it has given very satisfactory results.

The behavior of morphine in biological material such as animal tissues cannot be followed so easily. The estimation of morphine under such circumstances has received much attention and an extensive literature describes the work. The methods all depend ultimately upon the isolation of morphine in some comparatively pure form, and the presence of pseudomorphine would never interfere, because if present, it would be excluded by the various processes of protein removal, clarification, and alkaloid extraction which in one form or another invariably accompany all our morphine methods. Only a few attempts have been made, however, to measure the pseudomorphine. Marmé (1) sought to do this, apparently using methods resembling those for morphine, to which, for this purpose, objection has just been taken. Donath (2) was able to estimate both bases, but in urine only, and hardly with satisfying accuracy. In this laboratory a method of determining both morphine and pseudomorphine when together in biological material has been developed, but in such a complex mixture no way of estimating the amount of "higher oxidation products" has been successful. This is because proteins, proteoses, and some peptones are also precipitated with silicotungstic acid, while the prior removal of these interfering substances would here, as in the standard methods for morphine, carry off practically all the pseudomorphine, and much of the more highly oxidized group.

An account of the materials used in testing these methods is given below, followed by descriptions of the details in determining the individual bases. The scheme of separating the mixed silicotungstates is next outlined, and finally a description of the modification adapted to biological material is given.

Preparation of Materials.

Morphine.—Pure morphine was prepared from the commercial hydrochloride by a method designed primarily to eliminate pseudo-

morphine and the other oxidation products. The acetic acid solution was warmed with decolorizing carbon, and the base precipitated first by ammonia and secondly after solution in caustic potash, with acetic acid, adjusting the reaction to about pH = 9.0. Kolthoff (3) has determined the isoelectric point of morphine at pH = 8.96. The free base was redissolved in hydrochloric acid and salted out of this solution by adding about 5 volumes per cent of the concentrated acid. At this concentration the morphine hydrochloride exhibits a maximum insolubility. The hydrochloride was then dissolved and crystallized out of pure methyl alcohol, washed with ethyl alcohol, and dried under reduced pressure. A practically anhydrous morphine hydrochloride was obtained, with a specific rotation $[\alpha]_D = -96.4 \pm 0.4$ at 27° on a 2 per cent solution (calculated to a basis of $3H_2O$ of crystallization). On further recrystallization from water this rotation did not change appreciably. $[\alpha]_D = -95.9$ (2 per cent solution).

The free base was prepared from this hydrochloride by precipitation with ammonia, solution in boiling absolute alcohol, and separation from this solution by chilling. Washed with alcohol and vacuum-dried, it was a white, feathery, beautifully crystalline substance containing 6.22 per cent water, which corresponds to slightly more than 1 mol of water of crystallization.

Pseudomorphine.—The preparation of pseudomorphine hydrochloride has been described in a preceding paper.

Higher Oxidation Products.—A solution of the higher oxidation products of morphine was made by treating the slightly alkaline solution of commercial hydrochloride with successive additions of 3 per cent hydrogen peroxide for 24 hours on the water bath. On testing the dark colored solution, it was found to contain neither morphine nor pseudomorphine. It was then acidified with hydrochloric acid, evaporated to dryness, to remove hydrogen peroxide and free acid, dissolved in water with a small amount of potassium bicarbonate and filtered to remove a small amount of insoluble matter.

The morphine-derived substances in this solution were precipitated by silicotungstic acid, but the precipitate is more readily soluble in water than that formed from morphine itself. They were partially precipitated by strong acids, completely and readily

soluble in even very weak alkali. On evaporating the acid solution, these dark colored substances were partially soluble in the higher alcohols, chloroform, and benzyl alcohol; nearly insoluble in the liquid hydrocarbons, lower alcohols, and ether. From alkaline solutions, they were not removed by any of these solvents. These substances resemble morphine in their qualitative properties only by precipitability with most of the alkaloidal reagents, such as the derivatives of tungstic and molybdic acids, picrolonic acid, trichloroacetic acid, salts of lead, mercury, gold, platinum, and the like. They are obviously more acid in character than either morphine or pseudomorphine.

The precipitate with silicotungstic acid, when dried at 120° contains about 64 per cent silica and tungsten oxide, which is less than the value for the corresponding morphine precipitate.

TABLE I.

Determination of Morphine as Silicotungstate.

Sample No.	Morphine (free base).		Calculated from oxides.	Calculated from loss on igniting.
	Taken.	Found. Calculated from dried precipitate.		
	gm.	*gm.*	*gm.*	*gm.*
1	0.0815	0.0807	0.0807	0.0807
2	0.1630	0.1622	0.1640	0.1594
3	0.0818	0.0818		
4	0.0805	0.0815		

Determination of Morphine as Silicotungstate.

Bertrand (4) showed that morphine silicotungstate dried at 120° has a constant composition: $SiO_2 \cdot H_2O \cdot 12WO_3 \cdot 4$ morphine $+ 3H_2O$. As a means of estimating morphine it is open to two objections, appreciable solubility and a tendency to form colloidal suspensions. These difficulties are overcome by using solutions of small volume containing a strong electrolyte, in which the freshly formed precipitate is coagulated by shaking.

The morphine solution, if possible not more than 10 cc. in volume, is placed in a small test-tube, acidified with hydrochloric acid, and a few crystals of potassium chloride are added. An excess of acid, even amounting to 2 or 3 per cent, does no harm. An approxi-

mately 10 per cent excess of silicotungstic acid is added (as a 10 per cent solution in water). A yellowish pink precipitate forms at once. The tube is stoppered and vigorously shaken for several minutes, then chilled by immersing in cold water. The precipitate coagulates and may be filtered onto a Gooch mat. Some of the filtrate is used to wash out the tube. The morphine silicotungstate is washed with cold water containing 2 or 3 cc. of hydrochloric acid per liter, but on account of the solubility of the precipitate an excess of wash water is avoided. Alcohol should not be used as a washing fluid. The crucible is dried at 120° until weight is constant (usually 2 or 3 hours). The method has the advantage that only 0.2810 of the weighed precipitate is morphine (as free base).

As a check determination the crucible may be ignited, and the weight of the residual oxides of tungsten and silicon determined. Due to the hygroscopic nature of freshly ignited silica, this procedure needs care.

Morphine silicotungstate $3H_2O$ (as dried at 120°) $\times$ 0.2810 = free base.
Ignited oxides $\times$ 0.401 = free base.
Loss on ignition of the silicotungstate $\times$ 0.9406 = free base.

Working with pure morphine solutions, the method gives most excellent results, provided the volume of liquid is kept small.

Determination of Pseudomorphine.

No quantitative methods for the estimation of pseudomorphine have been described. The exceptional insolubility of many salts of this base makes several good methods possible. In this laboratory pseudomorphine has been determined in three forms, the free base, the silicotungstate, and the sulfate.

Determination as the Silicotungstate.—The precipitation of pseudomorphine silicotungstate can be carried out in the same manner as that of morphine silicotungstate, but the greater insolubility of the pseudomorphine compound makes it unnecessary to reduce the volume of the solution so much. The solution may be warmed. The precipitate is weighed on a Gooch mat after drying at 120°, but it may be safely washed with alcohol as well as with acidulated water, which facilitates the drying. The weighed precipitate may also be ignited as a check determination. The

precipitate when dried at 120° contains no water of crystallization, and the factors for calculating the free base are as follows:

Pseudomorphine × 0.283 = free base.
Ignited oxides × 0.399 = " "
Loss on ignition × 0.970 = " "

Determination as Free Base.—Pseudomorphine has a rather wide isoelectric range, and since the free base is highly insoluble, it may be precipitated by adjusting the reaction of the solution to a pH of about 7.8. The separated base is hard to filter unless electrolytes are present; a convenient method of obtaining the proper conditions is to make the solution decidedly alkaline with KOH or NH_4OH, and then add a large amount of solid ammonium chloride. After this dissolves, the solution should be acid to

TABLE II.

Determination of Pseudomorphine as Silicotungstate.

Pseudomorphine.	
Taken.	Found.
gm.	*gm.*
0.0218	0.0218
0.0436	0.0432
0.0436	0.0429
0.0872	0.0884

phenolphthalein, but still alkaline to litmus. The base precipitates very slowly and is crystalline. It may be filtered in a Gooch crucible, washed with 30 per cent alcohol, and dried to constant weight. Water cannot be used as a wash liquid, since the base comes through the filter in colloidal condition when all the salts are removed. Under these conditions the solubility of the base is about 2 mg. per 100 cc.; so the method is not recommended for small amounts.

Determination of Pseudomorphine as Mixture of Silicotungstate and Free Base.—If silicotungstic acid be added to the alkaline solution of pseudomorphine, and the reaction adjusted as described for the free base, a mixture of the free base and the silicotungstate separates. This precipitation is quantitative. In order to deter-

mine the pseudomorphine it is filtered off, washed with 30 per cent alcohol, and the loss, on ignition, in the weight of the dried precipitate is found. Considerable variations in the pH of the solution do us no harm, but merely change the ratio of free base to silicotungstate precipitated. Since the presence of ammonium salts may be objectionable, other substances having a buffer effect may be used to adjust the reaction. Glycocoll is satisfactory, and a solution of potassium dihydrogen phosphate works very well. With the last named substance, the solution is made slightly but distinctly alkaline to litmus. The advantage of this method lies in its ability to separate pseudomorphine from morphine, the higher oxidation products of morphine, and protein hydrolysis products.

TABLE III.

Determination of Pseudomorphine as Mixed Precipitate of Free Base and Silicotungstate.

Pseudomorphine (free base).		Buffer solution contained.
Taken.	Found.	
gm.	*gm.*	
0.0626	0.0638	NH_4Cl
0.0626	0.0607	
0.0624	0.0625	
0.0626	0.0622	KH_2PO_4
0.0624	0.0606	
0.0626	0.0614	Glycocoll.

When considerable silicotungstate occurs in the precipitate the loss on ignition must be corrected by the amount of combined water present. This may be calculated from the weight of the oxides remaining in the crucible after ignition.

Free base = loss on ignition of mixed precipitate − (0.013) × (weight of oxides remaining after ignition).

Determination as Sulfate.—Pseudomorphine sulfate is only slightly soluble in strong alcohol, and even less so in acetone. From either of these media, but particularly from the latter, it is quantitatively precipitated in the presence of sulfuric acid. The method is useful in separating pseudomorphine from alkaloids,

particularly morphine, which give soluble sulfates. The precipitate is brought into a Gooch crucible, washed with acetone, and dried to constant weight at 100°.

Pseudomorphine sulfate × 0.852 = free base.

Separation of Morphine, Pseudomorphine, and Higher Oxidation Products of Morphine.

After acidifying an oxidizing morphine solution and treating with silicotungstic acid, these substances are at hand as the silicotungstates. They are thus quickly removed from the reaction, and may be separated at leisure. When all three classes of constituents in this precipitate are to be estimated, it is necessary to work with two parallel samples, on one of which the total weight of precipitated silicotungstate is determined, while on the other the morphine and pseudomorphine are found. When the last two are known, the quantity of the higher oxidation products (as silicotungstates) can be calculated by difference. It is necessary then to determine morphine and pseudomorphine in the presence of each other and of the higher oxidation products.

The separation of pseudomorphine has been done by using the ammonium chloride or the potassium phosphate buffer solution previously described, which yields a mixture of the free base and the silicotungstate. Neither morphine nor its higher oxidation products interferes. Small amounts of protein and other foreign substances precipitable by silicotungstic acid are likewise harmless, but of course do prevent the parallel estimation of the higher oxidation products. The case where large amounts of protein are present, as in tissue, is discussed later.

The filtrate from the pseudomorphine contains the morphine which must be separated now from its oxidation products. This can be done by taking advantage of the more pronounced acidic character of the latter group and extracting the morphine from a comparatively alkaline solution.

Considerable objection has been made to the method of removing morphine by extraction, and it is liable to serious error unless the reaction of the solution is carefully regulated and kept constant during the extraction. Morphine is most easily extracted at its isoelectric point, approximately pH = 8.9. A variation from 8.9

TABLE IV.

Extraction of Morphine from Solutions of pH = 9.1 in a Continuous Extractor.

Morphine (base).		Extraction time.	Solvent.
Added.	Recovered.		
gm.	*gm.*	*min.*	
0.0815	0.0815	60	$CHCl_3 + C_2H_5OH$.
0.0815	0.0800	50	" "
0.0815	0.0820	40	Amyl alcohol.
0.0815	0.0817	60	" "
0.0815	0.0790	40	Butyl "

TABLE V.

Estimation of Pseudomorphine in Presence of Morphine and Its Oxidation Products.

	Morphine (free base).	Pseudomorphine (free base).	Higher oxidation products calculated as silicotungstate × 0.36.
	gm.	*gm.*	*gm.*
Taken	0.2	0.0626	0.05
Recovered		0.0660	
Taken	0.18	0.0626	0.05
Recovered		0.0660	
Taken	0.15	0.0626	0.03
Recovered		0.0631	
Taken	0.15	0.0624	0
Recovered		0.0600	
Taken	0.15	0.0624	0.03
Recovered		0.0612	
Taken	0.10	0.0624	0
Recovered		0.0616	
Taken	0.10	0.0626	0.015
Recovered		0.0632	

to 9.1 is quite allowable, but the solution retains considerable morphine if the variation becomes larger. This is very noticeable

with amyl alcohol, which is one of the best solvents for the alkaloid. A variation on the acid side is of less moment with the lower alcohols and chloroform as solvents, due to the solubility of morphine salts in the former, and the practice of mixing ethyl alcohol with the latter. Variation on the acid side of the isoelectric point will allow the oxidation products to be extracted also; while the older methods of using an excess of ammonia or soda hold back morphine because the solution is far too alkaline. The necessity for a heavily buffered solution, containing for instance much phosphate, is also quite apparent.

TABLE VI.

Results of Described Method for Estimating Morphine, Pseudomorphine, and Their Silicotungstic Acid-Precipitable Oxidation Products in Various Synthetic Mixtures.

	Morphine (free base).	Pseudomorphine (free base).	Higher oxidation products calculated as silicotungstate × 0.36.
	gm.	*gm.*	*gm.*
Taken	0.0163	0.0624	0.0860
Recovered	0.0164	0.0596	0.0876
Taken	0.0815	0.0624	0.0130
Recovered	0.0808	0.0612	0.0142
Taken	0.0165	0.0624	0.0860
Recovered	0.0173	0.0618	0.0846
Taken	0.0815	0.0124	0.0860
Recovered	0.0832	0.0112	0.0875

It is not generally realized that butyl alcohol and alcohol containing chloroform will extract large quantities of morphine from even strongly acid solutions, at least if continuous extraction is employed. With butyl alcohol as a solvent, 10 cc. of a (nearly) 1 per cent morphine hydrochloride solution, containing 1 per cent sulfuric acid, were extracted for 30 minutes in a continuous extractor. By this time 72 per cent of the morphine had been removed. In a similar experiment amyl alcohol removed only 9 per cent of the morphine in the same time. The last traces of the alkaloid are removed only with difficulty, however, and there seems no possibility of basing an analytical procedure on this behavior.

For the separation of morphine from its oxidation products, the filtrate from the pseudomorphine (evaporated if necessary) is treated with 2 or 3 gm. of disodium phosphate, and then sufficient potassium hydroxide to bring the reaction to a pH = 9.0 or 9.1. Because the solutions are frequently highly colored, this is best tested by bringing a drop of the solution in contact with a drop of dilute brom-thymol blue on a spot plate. Comparison with a known buffer solution makes a sufficiently accurate adjustment easy. The solution is placed in a continuous extractor, and may be extracted with amyl or butyl alcohol (under vacuum) or with chloroform containing about 5 per cent ethyl alcohol. Since extractors vary greatly in their efficiency, no time of extraction can be specified. With the "lighter than water" extractor used in this laboratory, 0.1 gm. of morphine dissolved in 10 to 15 cc. of solution is completely removed by amyl alcohol in 35 minutes. The solvent is evaporated on the water bath, being replaced with water containing a few drops of hydrochloric acid. Usually traces of waxy substances are present and can be removed by shaking the acid solution once or twice in a separatory funnel with pure chloroform or benzene. The acid solution is then evaporated, nearly to dryness, filtered if necessary, and the morphine in this small volume of liquid is determined as already described by the silicotungstate precipitation.

Determination of Morphine and Pseudomorphine in Tissue.

As stated previously only the determination of morphine and pseudomorphine is considered, not that of the higher oxidation products.

The method was worked out on ground beefsteak containing a considerable amount of fat. It is not claimed to be applicable to material differing greatly from this. Weighed amounts of the powdered bases were added to the meat, and then thoroughly incorporated by grinding with sand.

The separation of these bases from tissue is rendered difficult by the similarity between proteoses and pseudomorphine in their behavior toward precipitants. It may be made from small amounts of proteose, peptone, and the like, by precipitating the free pseudomorphine at its isoelectric point, or by precipitating the mixture of free base and silicotungstate at a pH of 7.3 to 7.5.

XUM

With fairly large quantities of pseudomorphine, the former procedure is satisfactory, but with only a few mg., the protein acts as a protective colloid, and interferes with the precipitation of the base, so that the alternative method must be used.

It is necessary first to remove the bases to be determined from the greater part of the tissue constituents. This is done by taking advantage of the solubility of both morphine and pseudomorphine in benzyl alcohol. At the pH of ordinary tissue fluids, pseudomorphine is present entirely as the free base.

From 10 to 30 gm. of meat are thoroughly disintegrated in a mortar with a known weight of sand, and then transferred to a weighed flask. About 50 cc. of absolute alcohol are added, and the mixture shaken until thoroughly uniform. It is allowed to stand for a few minutes until dehydration of the shreds of tissue is complete, then 100 to 125 cc. of benzyl alcohol are added. The shaking is repeated, and the contents of the flask heated to about 70° by immersing in warm water. The tissue should now present a rather translucent and uniform appearance and all fat should be dissolved. The flask is allowed to stand until cool, and again weighed, in order to determine the exact amount of material (exclusive of sand) which it contains. An aliquot part of this material is taken by weighing the solvent filtered off through a dry hardened filter paper into a tared flask. This aliquot generally represents about 80 per cent of the total. A correction may be made for the weight of the anhydrous tissue present in the solvent. This method is easier than the corresponding volumetric procedure, when handling a substance as viscid as the benzyl alcohol mixture. The expense and difficulty of handling large amounts of benzyl alcohol make the more accurate method of exhaustive extraction impracticable.

Besides its aliquot of the alkaloids, the solvent contains fat, very small amounts of protein, and coloring matter. The bases are next extracted from the solvent by dilute hydrochloric or acetic acid. Since the specific gravity of benzyl alcohol is near that of water, the solvent is mixed with an equal volume of benzene which decreases its specific gravity and also lessens the solubility of the bases in the non-aqueous phase during extraction. The extraction is made repeatedly in a funnel with ½ per cent hydrochloric acid until the extract gives no opalescence with phosphotungstic acid. One or

two extractions are then made with distilled water, all the extracts united, and washed by shaking twice or thrice with benzene. If the water layer is not clear, and it usually is not, it is filtered through hardened paper. This extraction should be thorough, and very dilute acid must be used, because of the insolubility of pseudomorphine salts in acid. No trouble is caused by permanent foam or emulsion.

The acid extract is next evaporated to small volume, never over 15 cc. It should be at the most only slightly yellow. It is brought into a small beaker, cooled, a few crystals of potassium chloride added, and then sufficient silicotungstic acid for complete precipitation. After the precipitate, which may become sticky, has collected together, it is filtered, washed with a little 0.2 per cent hydrochloric acid, and then dissolved off the filter with a few drops of warm dilute potassium hydroxide solution. The volume should not be more than 15 or 20 cc., unless large amounts (50 mg. or more) of morphine are present.

The pseudomorphine is next precipitated, by first neutralizing the liquid to phenolphthalein with hydrochloric acid and then adding a solution of KH_2PO_4 drop by drop until the liquid is just slightly but definitely alkaline to litmus paper. The mixture of pseudomorphine and its silicotungstate is allowed to stand until the supernatant fluid is quite clear, and then handled as previously described. If the quantity of precipitate is very small, instead of filtering on a Gooch mat it is better to use a circle of hardened filter paper, wash as usual with 30 per cent alcohol, then redissolve in a little alkali, add a drop of silicotungstic acid solution, and precipitate the pure silicotungstate with hydrochloric acid. This more bulky precipitate is easily filtered in a Gooch crucible, dried, and weighed.

The filtrate from the pseudomorphine contains some peptones, as well as the morphine. It is handled by extraction with chloroform or amyl alcohol, precisely as described for the separation of morphine and its oxidation products.

Table VII shows the results obtained by this method from a series of consecutive experiments.

The results are, in general, gratifyingly low. There seems to be little chance of impurities leading to high results. In the case of pseudomorphine, the greatest single error apparently lies in

incomplete extraction from the benzyl alcohol. With morphine, however, there is danger of loss by oxidation while carrying out the method. It is not wise to prolong the analysis unduly, nor to allow alkaline solutions to stand in the air longer than necessary, as they are more easily oxidized than when acid. The presence of ammonium salts is to be avoided because of the difficulty in maintaining a constant alkalinity during extraction of the morphine. As a method for morphine alone, the procedure is not bad, and is rather more rapid than those ordinarily in use.

The author wishes to thank Dr. L. A. Ryan of this faculty for several helpful suggestions, particularly regarding this phase of the work.

TABLE VII.

Recovery of Morphine and Pseudomorphine from Ground Meat.

Sample No.	Meat.	Pseudomorphine.		Morphine.	
		Taken.	Recovered.	Taken.	Recovered.
	gm.	*mg.*	*mg.*	*mg.*	*mg.*
1	12	17.3	*	2.6	2.0
2	14	3.3	3.7	17.6	*
3	24	15.9	15.6	10.5	7.9
4	21	16.6	15.1	9.3	6.6
5	25	7.2	5.1	1.7	1.9
6	26	1.4	1.4	2.5	1.2

* Not estimated.

SUMMARY.

Pseudomorphine may be quantitatively determined in several forms, which make possible a method for the chemical separation and analysis of a morphine solution which is undergoing oxidation. The reaction may be abruptly terminated and the composition of such a system determined at any given time with respect to residual morphine, pseudomorphine, and the other morphine-derived substances precipitable by silicotungstic acid, taken together as a class. If desired, five variables can thus be evaluated in the system, when the initial concentration of morphine and the quantity of oxidant used are also known.

A modification of the method enables the estimation of morphine

and pseudomorphine in amounts as small as 2 mg. in 20 to 30 gm. of biological material like ground meat, with an error of about 30 per cent. In the analysis of tissues for morphine alone, the method is also useful, because rapid.

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THE PURIFICATION OF PEPSIN, ITS PROPERTIES, AND PHYSICAL CHARACTERS.

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Since 1836, when Theodor Schwann signalized the presence in gastric juice of the proteolytic ferment which he was the first to designate as pepsin, a large number of attempts have been made to isolate it in a pure form to determine its chemical nature and its ultimate physiological properties. In a large majority of these, and, especially, all those made before 1895, except that of Brücke (1), who was the first to use adsorption, the methods used would not now be regarded as adequate for the purpose, and the results they gave are, accordingly, now only of historic interest in relation to the subject. In consequence a detailed reference to them will be omitted here and only such citations of the later publications to which we owe what at present we know about pepsin will be made in this contribution on the subject.[1]

Pekelharing (2), by dialyzing, at low temperature, against distilled water, gastric juice from a dog with a Pavlov fistula, till the concentration of hydrochloric acid in the juice was decreased to about 0.02 per cent, precipitated the pepsin therein as a finely divided suspension. This suspension he separated by centrifuging and, after washing the sediment with distilled water, dried it in a desiccator. From the clear fluid left after centrifuging he precipitated, by half saturation with ammonium sulfate, more pepsin which was then freed from this salt by dialysis and, after the addition of hydrochloric acid to the extent of 0.2 per cent, was subjected to dialysis against distilled water, thus furnishing an additional quantity of pure pepsin. The average composition of the pepsin so prepared was C 51.99, H 7.07, N 14.44, S 1.63, Cl 0.49, P 0.01 per cent.

The phosphorus found in pepsin Pekelharing regarded as due to traces of an impurity resulting from the digestion of a phosphorus-containing protein. The chlorine, on the other hand, he found to be constant, even in preparations obtained by dialysis with oxalic acid.

He obtained a coagulated product of an acid character on heating either gastric juice or purified pepsin in acid solution, to which he gave the name

[1] An extended account of the literature of the subject is given in Oppenheimer, K., Die Fermente und ihre Wirkungen, Leipsic, 5th edition, 1925.

peptic acid. It was soluble in alkaline solutions but insoluble in dilute acids. It was soluble in water and in hot alcohol. It gave the biuret, Millon's, and Adamkiewicz's reactions but, when in solution, did not give off its sulfur on heating with alkali. Its chemical composition was C 50.79, H 7.02, N 14.44, S 1.08 per cent.

Nencki and Sieber (3), by dialyzing at a low temperature fresh gastric juice from a gastric fistula made by Pavlov's method, obtained a precipitate which contained chlorine, iron, and phosphorus, and which on heating with hydrochloric acid yielded a nucleoprotein and an albumose. The nucleoprotein on hydrolysis with sulfuric acid yielded purine bases and pentoses. On washing the precipitate with alcohol, the chlorine was decreased and a lecithin-like substance was extracted. It was found that the content of iron and phosphorus was inconstant, but, nevertheless, they believed they had isolated pepsin in a state of purity, that the inconstancy of its composition was due to its labile character, and that its molecule consists of a nucleoprotein, lecithin, phosphoric acid, chlorine, and iron. The ultimate analysis of their pure product gave the following values: C 51.26, H 6.74, N 14.33, S 1.20, Cl 0.476, P 0.104 per cent.

The ultimate composition of pepsin, as determined by Pekelharing and Nencki and Sieber on their preparations of it, would appear to indicate that it is a protein. Ringer (4), however, holds that in the purest preparation of pepsin which he made by dialysis there was a mixture of protein and the enzyme proper as he found that in the electric field the former migrated to the cathode while the pure product, which was very actively digestive, passed always to the anode and had no isoelectric point. He did not prepare any of this product for analysis, and its composition was, therefore, undetermined, but he found his purest preparations free from chlorine and iron. Hammarsten (5), by the use of methods which gave him preparations of pepsin as active as those obtained by Pekelharing, but of which he made no elementary analyses, generalized from his observations on these that pure pepsin is always an enzyme-protein compound. Davis and Merker (6), by salting out aqueous solutions of commercial pepsin, filtering, and dialyzing, obtained very active preparations of pepsin (1:40,000 as compared with 1:3000 U.S.P. standard) which contained 2.01 per cent mineral matter, no chlorine, 0.47 per cent P_2O_5, 1.01 per cent CaO, and 1.5 per cent S. The nitrogen content was 13.77 per cent and that in α-amino acid form was only 0.61 per cent. Of the protein reactions the Molisch was marked and this, with the other characters manifested by the product, led Davis and Merker to regard pepsin as a glycoprotein.

Effront (7), by using paper pulp made from certain types of Dreverhoff's filters (No. 311) as an adsorbent, obtained very active preparations of pepsin which contained only 0.4 per cent nitrogen and with an inorganic (ash) content of 1.6 per cent. He reported no analyses of the product.

From this all too brief review of the literature of the subject it will appear that the chemical and other characters of pepsin are as yet not definitely determined and that further research is

necessary in order to establish what pepsin is. It was with the hope of adding somewhat to our knowledge of its chemical constitution and physical properties that the author undertook an investigation on it, and he now presents for record the results which he has obtained.

Methods of Investigation and the Results.

The investigation was directed to deal with the following:

1. Method of standardization of the digestive power of pepsin.
2. The purification of pepsin involving: (*a*) The determination of a suitable adsorbent; (*b*) adsorption with aluminium hydroxide and subsequent liberation and precipitation; (*c*) precipitation with safranine and removal of the safranine with isoamyl alcohol.
3. Chemical analyses of the various preparations.
4. (*a*) Hydrolysis of purified pepsin and determination of the various amino acids and purine bases resulting as well as the nitrogen distribution therein; (*b*) color reactions given by the various preparations.
5. Determination of the isoelectric point.
6. Influence of pepsin on the conductivity and hydrogen ion concentration of acid solutions of the same.
7. Effect of extraction with ether on the activity of pepsin.
8. Effect of alcohol-ether mixtures on solutions of pepsin at various hydrogen ion concentrations.

1. Method Employed in Standardizing Peptic Activity.

The method used in determining the peptic activity of the various preparations is that commonly known as the Mett tube method. A series of glass tubes, from 1 to 2 mm. in diameter, was filled with fresh white of egg and the albumen coagulated at about 90°C. The tubes were cut as required into fragments 10 to 15 mm. in length, and two of these placed in the enzyme liquid to be examined. The preparations were then placed in an incubator at about 37°C. for 24 hours, after which the tubes were removed and the length of albumen dissolved at both ends carefully measured. The temperature of the incubator varied considerably from one determination to another. However,

since each complete series was carried on simultaneously, all the results of such a series can be compared, but on account of the fluctuations of the temperature from time to time, the results of different series are not necessarily comparable.

Schutz (8) and Borissow (9) investigated this method and they found that within certain limits the rate of digestion, or the lengths of the column of albumen dissolved in the same time interval, is proportional to the square root of the quantity of enzyme. In other words, if M and M^1 are the lengths of albumen dissolved in the same time interval, the ratio of their concentrations is $1: \left(\frac{M^1}{M}\right)^2$.

2. Purification of Material.

(*a*) Preliminary experiments were carried out on the adsorption of pepsin by various organic and inorganic adsorbents in the hope that one would prove satisfactory. The various adsorbents used were animal charcoal, kaolin, Lloyd's reagent, cholesterol, camphor, salicylic acid, acetanilide, aluminium oxide, and freshly precipitated aluminium hydroxide. The results obtained from these determinations showed that cholesterol, camphor, salicylic acid, acetanilide, and aluminium oxide adsorb only traces of the active principle; kaolin and animal charcoal adsorb it partially from both neutral and acid solutions; Lloyd's reagent adsorbs it slightly from neutral solutions but very markedly from acid solutions. The pepsin adsorbed by Lloyd's reagent could be liberated only with great difficulty, consequently this reagent was not applicable for the preparation of the enzyme in a pure form. Freshly precipitated aluminium hydroxide, on the other hand, was found to be not only a very efficient adsorbent but the adsorbed pepsin could be readily liberated by dissolving the aluminium hydroxide in dilute hydrochloric acid. This adsorbent was therefore used in one of the methods of purification employed. The results obtained with it are given below.

Adsorption with Freshly Precipitated Aluminium Hydroxide.

Experiment 1.—2 gm. of aluminium chloride were dissolved in 100 cc. of distilled water and the aluminium precipitated, in the cold, with concentrated ammonium hydroxide. The precipitated

aluminium hydroxide was centrifuged off and washed several times with distilled water. It was then added to a solution of pepsin in distilled water (10 gm. of pepsin in 150 cc. of water). The solution was first distinctly acid to litmus, but on standing for a few hours in contact with the aluminium hydroxide the hydrogen ion concentration of the solution decreased to pH 4.5 ±0.3. The precipitate was then centrifuged off and the activity of the supernatant liquid compared with that of the original solution. 1 cc. of each solution was diluted with 7.5 cc. of 0.2 per cent hydrochloric acid. The digestive power of these diluted solutions was then determined with Mett tubes.

	Digestion. *mm.*	Ratio of concentrations.
Original solution	9.0	100
Supernatant liquid	2.5	7.8
Amount adsorbed	92.2 per cent.	

Experiment 2.—1 gm. of aluminium chloride was dissolved in 75 cc. of water. The aluminium was precipitated with ammonium hydroxide and washed as described in Experiment 1. The aluminium hydroxide was then added to a solution of pepsin in water (10 gm. of pepsin in 150 cc. of water) and allowed to stand, with occasional shaking, for several hours. The aluminium hydroxide was then centrifuged off and the activity of the supernatant liquid compared with that of the original solution.

	Digestion. *mm.*	Ratio of concentrations.
Original solution	8.0	100
Supernatant liquid	4.0	25
Amount adsorbed	75 per cent.	

Experiment 3.—5 gm. of pepsin were dissolved in 75 cc. of N/10 HCl and about 5 gm. of freshly prepared and washed aluminium hydroxide added. The acidity of the solution fell in a couple of hours to pH 4.5 ±0.3 on account of the basic action of the aluminium hydroxide. After centrifuging off the aluminium hydroxide, the digestive action of the supernatant liquid was compared with that of the original solution.

	Digestion. *mm.*	Ratio of concentrations.
Original solution	10	100
Supernatant liquid	1.25	1.50
Amount adsorbed	98.5 per cent.	

XUM

(*b*) *Preparation by Adsorption with Aluminium Hydroxide.*—Artificial gastric juice was prepared in the usual manner by digesting finely minced stomach mucosa of pigs, for several days, in 0.4 per cent hydrochloric acid and then filtering through a thick layer of paper pulp. The filtrate though yellow was perfectly clear. About 20 gm. of freshly precipitated and washed aluminium hydroxide were then added per 4 liters of solution. After a few hours, with occasional shaking, the supernatant liquid was decanted and the aluminium hydroxide separated by centrifuging. It was then placed in collodion dialyzing tubes and dissolved by dialyzing against 0.5 per cent hydrochloric acid for several days. A fairly large residue of organic material remained undissolved and was filtered off. The filtrate was dialyzed against tapwater until a small precipitate formed.[2] This precipitate was centrifuged off and the dialysis continued against tap water until a fairly large precipitate separated out. This precipitation reached a maximum at about pH 3.5. The digestive power of the precipitates was determined in various samples prepared and compared with that of standard pepsin 1:3000 U.S.P. The first precipitate never exhibited more than a very slight digestive action, but the second showed activities varying from 1:40,000 to 1:42,000 U.S.P. On several occasions this precipitate was snow-white but it had generally a slightly yellow color. This preparation was analyzed and will be referred to as Preparation I.

Pepsin prepared in the manner described above was further purified as follows: It was dissolved in 0.2 per cent hydrochloric acid and dialyzed against a large volume of distilled water. A small portion of the active principle was thus precipitated, but the main portion remained in solution. After centrifuging off the precipitate, the remainder of the active principle was precipitated by adding a small quantity of a neutral lead acetate solution. The precipitate was centrifuged off after a few hours and dissolved in a small volume of a saturated oxalic acid solution. The lead oxalate precipitate which resulted was filtered off and the filtrate dialyzed against distilled water. The dialyzing solution was

[2] Tap water was used for the dialysis as it was found to give better results than distilled water. Although the solution became very opalescent when distilled water was used as the dialyzing medium only a very small amount of the active principle could be removed by centrifuging.

changed several times and the dialysis continued until no traces of oxalates could be detected in the pepsin solution. A small precipitate generally formed during the dialysis; this was transferred with the solution to a large watch-glass, evaporated at room temperature, and finally dried in a desiccator over sulfuric acid. Preparations made in this manner varied from 1:39,000 to 1:45,000 U.S.P. This preparation will be referred to as Preparation II.

Although this method of purification decreased the percentage of ash and gave a very active product, it cannot however be considered as wholly satisfactory. There was always a great loss of the active principle during the dialysis. In some cases this loss reached to about 75 per cent of the original material. In the final stages of the dialysis, when the hydrogen ion concentration of the solution was low, the dialyzing liquid generally became opalescent although it manifested only very slight digestive action. It is possible that the enzyme, even at the hydrogen ion concentration of distilled water, may be broken down into fractions which would pass through collodion membranes. The removal of the fractions by dialysis would enable the decomposition to proceed to a great degree, and this would explain the great loss in material during the treatment, but with no corresponding increase in the digestive power of the preparation. Losses of like magnitude were obtained by simply dissolving the pepsin in 0.2 per cent hydrochloric acid, dialyzing against distilled water for several days, and evaporating the solution to dryness at room temperature.

(c) *Precipitation with Safranine.*—Marston (10), in 1923, showed that all the proteolytic enzymes were quantitatively precipitated from water solutions by safranine and induline, water-soluble dyes. He made quantitative determinations on the proteolytic activity of his trypsin dye preparation, but he does not appear to have determined the activity of his pepsin preparations. He made, it would seem, no serious attempt at separating the dye from the enzyme. In view of these results of Marston, it was decided to find out if this method could be employed in the purification of pepsin.

20 gm. of commercial pepsin were dissolved in about 350 cc. of distilled water and about 50 cc. of a 1 per cent solution of safra-

nine added. The pepsin was thus quantitatively precipitated. This precipitate was centrifuged off and washed several times with water by centrifuging. Its proteolytic activity was determined after drying and found to be 1:46,000 U.S.P.

Although this material gave very strong digestive action it was very insoluble in acid. In some cases it required almost 24 hours to dissolve 4 mg. in 10 cc. of 0.2 per cent hydrochloric acid. In the subsequent experiments the freshly precipitated pepsin-dye preparation was washed several times, by centrifuging, with approximately 0.1 per cent dye solution and finally with distilled water. The residue was then transferred, without drying, to a beaker and dissolved in a very weak oxalic acid solution in 20 per cent alcohol.

The dye can be almost completely removed from these solutions by repeated extractions with butyl or isoamyl alcohol. Butyl alcohol, however, is not satisfactory because in concentrated enzyme solutions an insoluble precipitate separates out which probably is altered pepsin. Isoamyl alcohol containing about 10 per cent ether gave quite satisfactory results. When the dye extraction was complete, the isoamyl alcohol which had dissolved in the pepsin solution was removed by repeated shaking with ether containing about 10 per cent ethyl alcohol. The pepsin was then precipitated from the solution by the addition of twice its volume of a mixture of equal volumes of ethyl alcohol and ether. The optimum pH for precipitation appears to be in the neighborhood of 2.5 and corresponds to the determined isoelectric point.

This method of preparation gave quite satisfactory results. From 100 gm. of crude commercial pepsin 3 gm. of purified material were obtained which gave a digestive action of 1:64,000 U.S.P. when compared with 1:3000 commercial pepsin. This corresponds to a recovery of about 65 per cent of the active principle present in the original material. This preparation will be referred to as Preparation III.

3. Chemical Analysis.

Preparation I.—(Aluminium hydroxide method.)

Ash.—0.2031 gm. of pepsin gave 0.0067 gm. of ash; therefore ash = 3.3 per cent.

Nitrogen Determination by Kjeldahl Method.

Determination No.	Weight of sample.	Weight of N.	N
	gm.	*gm.*	*per cent*
1	0.0898	0.0114	12.70
2	0.1028	0.0126	12.26
3	0.0830	0.0105	12.64
4	0.1008	0.0126	12.50
Average			12.52
Percentage when calculated on an ash-free basis			12.94

Carbon and Hydrogen by Combustion.

Determination No.	Weight of sample.	Weight of H_2O.	Weight of CO_2.	H	C
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
1	0.1222	0.0748	0.2040	6.80	45.53
2	0.1351	0.0841	0.2285	6.92	46.13
Average				6.86	45.83
Percentage when calculated on an ash-free basis				7.09	47.39

The analysis of Preparation I may be briefly summarized as follows: C 47.39; H 7.09; N 12.94.

Preparation II.—(Aluminium hydroxide and lead acetate method.)

Ash.—0.0889 gm. of pepsin gave 0.0004 gm. of ash; therefore ash = 0.45 per cent.

Nitrogen Determination by Kjeldahl Method.

Determination No.	Weight of sample.	Weight of N.	N
	gm.	*gm.*	*per cent*
1	0.0815	0.0111	13.62
2	0.0920	0.0124	13.48
3	0.1027	0.0138	13.44
4	0.0986	0.0131	13.29
Average			13.46

Carbon and Hydrogen by Combustion.

Determination No.	Weight of sample.	Weight of H_2O.	Weight of CO_2.	H	C
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
1	0.1433	0.0852	0.2555	6.61	48.63
2	0.1286	0.0808	0.2297	6.98	48.72
3	0.1302	0.0815	Lost.	6.95	
Average				6.84	48.67

Sulfur Precipitated as Barium Sulfate.

Determination No.	Weight of sample.	Weight of $BaSO_4$.	S
	gm.	*gm.*	*per cent*
1	0.1194	0.0092	1.06
2	0.2172	0.0168	1.06
3	0.2085	0.0152	1.00
Average			1.04

Phosphorus Precipitated as Magnesium Ammonium Phosphate.

Determination No.	Weight of sample.	Weight of $Mg_2P_2O_7$.	Weight of $Mg_2P_2O_7$ corrected.	P
	gm.	*gm.*	*gm.*	*per cent*
1	0.2172	0.0031	0.0022	0.29
2	0.2085	0.0028	0.0019	0.25
Blank.		0.0011		
"		0.0007		
Average				0.27

The analysis of Preparation II may be briefly summarized as follows:

	per cent		*per cent*
Ash	0.45	N	13.46
C	48.67	S	1.04
H	6.84	P	0.27

Preparation III.—(Safranine precipitation method.)

Ash.

Determination No.	Weight of sample.	Weight of ash.	Ash.
	gm.	*gm.*	*per cent*
1	0.1402	0.0017	1.21
2	0.1542	0.0018	1.16
Average			1.19

Iron.—The ash was dissolved in a small volume of concentrated hydrochloric acid, the iron precipitated by ammonia, filtered, ignited, and weighed as Fe_2O_3.

	Sample 1.	Sample 2.
	gm.	*gm.*
Weight of sample	0.1402	0.1542
" " ash	0.0017	0.0018
" " Fe_2O_3	0.0009	0.0009

Average per cent of iron in total sample, 0.43

Nitrogen by Kjeldahl Method.

Determination No.	Weight of sample.	Weight of N.	N
	gm.	*gm.*	*per cent*
1	0.1487	0.01706	11.47
2	0.0870	0.01008	11.59
3	0.0918	0.01017	11.08
Average			11.37
Percentage calculated on an ash-free basis			11.50

Carbon and Hydrogen by Combustion.

Determination No.	Weight of sample.	Weight of H_2O.	Weight of CO_2.	H	C
	gm.	*gm.*	*gm.*	*per cent*	*per cent*
1	0.1007	0.0555	0.1647	6.12	44.60
2	0.1090	0.0600	0.1790	6.12	44.79
Average				6.12	44.70
Percentage calculated on an ash-free basis				6.19	45.24

Sulfur Precipitated as Barium Sulfate.

Determination No.	Weight of sample.	Weight of $BaSO_4$.	S
	gm.	*gm.*	*per cent*
1	0.0960	0.0128	1.82
2	0.1260	0.0158	1.72
Average			1.77
Percentage calculated on an ash-free basis			1.79

The analysis of Preparation III, calculated on an ash-free basis, may be briefly summarized as follows:[3]

	per cent		per cent
C	45.24	N	11.50
H	6.19	S	1.79

4. (a) Hydrolysis of Purified Pepsin and Determination of Various Amino Acids and Purine Bases Resulting as Well as Nitrogen Distribution Therein.

The Andersen and Roed-Müller (11) modification of Van Slyke's (12) method for determining the nitrogen distribution

TABLE I.

Group.	Weight of N.	Per cent of total N.
	mg.	
Total N	505.0	100
" humin N	30.24	5.99
Amide N	46.06	9.12
Arginine N	14.71	2.91
Histidine N	17.76	3 52
Lysine N	5.02	1.00
Dicarboxylic acid N	18.13	3.59
Proline N	28.49	5.64
Amino N of monoamino acids minus dicarboxylic acid N	317.53	62.88
Total		94.65

in proteins was followed for these determinations. This method is given in detail by Plimmer (13) and consequently need not be given here. The results obtained from these determinations are briefly recorded in Table I.

Hydrolysis and Isolation of Resulting Amino Acids.—6.5 gm. of pepsin Preparation II were hydrolyzed with sulfuric acid and the

[3] This preparation was slightly colored from traces of the dye. Analyses of the safranine used were carried out by the Kjeldahl method, but the nitrogen obtained was only about one-half of the theoretical value. In all probability the nitrogen of the amino groups was alone converted in the digestion. Consequently the nitrogen value obtained for the pepsin may be slightly low. However, it should be noted that in the analysis of the pepsin the carbon remains low; if much safranine was present it would undoubtedly be high, because safranine contains about 73 per cent carbon.

hydrolysate divided into various fractions by Dakin's (14) butyl alcohol method. A number of amino acids was isolated from these various fractions and identified microscopically. The usual method of separation and identification of the diamino and dicarboxylic acid was employed. On account of the small amount of material available it was not found possible to separate definitely the various monoamino-monocarboxylic acids; tyrosine alone was isolated. It was present in fairly large amounts. The various amino acids isolated were: tyrosine, proline, glutamic acid, aspartic acid, histidine, lysine, and possible traces of arginine.

Determination of Purine Bases.—2 gm. of pepsin were hydrolyzed with dilute sulfuric acid and from the hydrolysate infinitesimal traces of purine bases were isolated by precipitation with ammoniacal silver nitrate. The precipitate gave the nitric acid test for xanthine or guanine, but the Wheeler-Johnson (15) reaction for uracil and cytosine was negative.

(*b*) *Color Reactions Given by Preparations II and III.*

Pepsin Preparations II and III were examined for the various protein color reactions, and positive results were obtained with the following tests: Biuret, Millon's, xanthoproteic, Pauly's diazo, Hopkins-Cole, Molisch, and Elliott and Macallum (16). Preparation II, but *not* Preparation III, reacted positively with the ninhydrin test.

5. Isoelectric Point of Pepsin.

Iscovesco (17), experimenting on the cataphoretic migration of pepsin in artificial and natural gastric juice, obtained results indicating that pepsin in hydrochloric acid concentrations of about 0.4 per cent migrates solely towards the cathode. Michaelis and Davidsohn (18) from similar experiments obtained a hydrogen ion value of 5.5×10^{-3} for the isoelectric point of Grübler's commercial pepsin. Pekelharing and Ringer (19), on the other hand, were not able to demonstrate the presence of an isoelectric point when they used pepsin preparations made according to Pekelharing's method. The pepsin migrated towards the anode at all hydrogen ion concentrations of the pepsin solutions.

In view of these conflicting results in regard to the isoelectric point of pepsin, it was considered of interest to determine the isoelectric point of two of the preparations obtained by the author, namely Preparations II and III. These two preparations were made in different ways and one of them had a digestive action approximately 60 per cent greater than the other. A comparison of the digestive powers of these preparations with those obtained by previous investigators indicates that our most active preparation was purer than any which had been previously obtained. This is further supported by a comparison of their chemical analysis.

These two preparations gave practically the same result in regard to the isoelectric point of pepsin. They both manifested anodic migration at pH 3 and above. Although in no case does either show pure cathodic migration, both show a definite preponderance in cathodic migration at a pH of about 2. This would give pepsin an isoelectric point of about 2.5. This value agrees fairly well with the Michaelis and Davidsohn value of pH 2.26 for Grübler's pepsin.

The apparatus used in these determinations was similar to the ordinary cataphoresis apparatus. The pepsin solution was placed in the central chamber and the side chambers filled with the same solution minus the pepsin, the arrangement of the apparatus being briefly thus:

Cu in $CuCl_2$ solution.	Solution.	Pepsin solution.	Solution.	Ag in dilute HCl.

The voltage was 110.

At the conclusion of an experiment the stop-cocks separating the pepsin solution from that of the side chambers were closed, and a portion of the solution in the side chambers siphoned off. Only the top portion of the solutions was removed, so as to overcome the chance of error from diffusion of the pepsin into the side chambers during the experiment. This was done by filling a small siphon with water with a stop-cock at one end; the other end was then placed about half-way to the bottom of the liquid in the side chamber. On opening the stop-cock the top portion of the liquid was siphoned off. Approximately equal volumes were thus removed from the cathode and anode chambers and

TABLE II.

Preparation II. Migration of Pepsin under Influence of an Electric Current.

Determination No.	Normality.		Digestion.		Ratio.	Duration.	pH
	Before.	After.	Cathode.	Anode.			
			mm.	mm.		hrs.	
1	0.044		1.85	2.15	1.0 : 1.2	4	
2		0.048	1.70	2.0	1.0 : 1.2	7	1.36*
3	0.0225	0.0226	2.75	2.75	1.0 : 1.0	12	
4	0.0225		3.25	3.25	1.0 : 1.0	10	
5	0.0225		2.85	3.00	1.0 : 1.1	6	
6	0.0225	0.0224	1.75	1.50	1.2 : 1.0	6	1.65*
7	0.0136		8.0	4.0	2.0 : 1.0	12	
8	0.0136		4.75	2.1	2 3 : 1.0	6	
9	0.0136	0.0139	3.75	2.75	1.4 : 1.0	6	1.87*
10	0.0086		1.75	1.0	1.75: 1.0	6	
11	0.0086		6.0	3.0	2.0 : 1.0	9	
12	0.0086	0.0090	3.25	1.75	1.8 : 1.0	6	2.07*
13	pH 2.2		4.5	3.5	1.3 : 1.0	9	
14	" 2.2	pH 2.1	2.15	1.75	1.3 : 1.0	6	2.2
15	" 2.4		1.65	1.65	1.0 : 1.0	9	
16	" 2.4		2.0	1.75	1.1 : 1.0	6	
17	" 2.4	pH 2.4	1.7	1.5	1.1 : 1.0	6	2.4
18	" 2.6		2.15	3.15	0.68: 1.0	11	
19	" 2.6		1.50	1.75	0.85: 1.0	6	
20	" 2 6		1.35	1.80	0.75: 1.0	7	
21	" 2.5	pH 2.5	4.0	4.0	1.0 : 1.0	12	2.6
22	" 3.0		0	1.60	0 : 1.6	5	
23	" 3.0		0	2.00	0 : 2.0	4	
24	" 3.0	pH 3.0	0	2.5	0 : 2.5	11	3.0
25	" 4.6		0	2.5	0 : 2.5	4	4.6
26		pH 3.6	0	1.75	0 : 1.75	4	3.6
27	0.0225		0.25	2.5	1.0 :10	24	
28	0.0225	0.0225	0.15	1.35	1.0 : 9	12	1.36†

TABLE II—*Concluded.*

Determination No.	Normality.		Digestion.		Ratio.	Duration.	pH
	Before.	After.	Cathode.	Anode.			
Blank experiments for diffusion, no current.							
			mm.	*mm.*		*hrs.*	
29	pH 4.6		0	0	0 : 0	6	
30	" 4.6	pH 4.6	0	0	0 : 0	14	4.6
31	0.0225	0.0225	0	0	0 : 0	24	1.65

* Calculated assuming complete ionization.
† Water used in side chambers.

consequently the digestion given by each is a close approximation of their relative activities. An equal volume of 0.2 per cent hydrochloric acid was added to the solutions and the peptic activity of these diluted solutions determined with Mett tubes, and expressed as mm. of albumen digested. Either the normality or pH of the pepsin solutions was determined before and after each series. The normality was determined by titration and the pH colorimetrically.

Experiment 1. Isoelectric Point of Preparation II.—In Determinations 1 to 17 (Table II) hydrochloric acid was used in making up the different pepsin solutions; in Determinations 18 to 21, lactic acid; in Nos. 22 to 24, acetic acid (approximately N/10); and in Nos. 25 and 26, acetic acid and sodium acetate (approximately 1 volume of N/10 acetic acid to 7 volumes of N/10 sodium acetate).

These results show a maximum cathodic migration at approximately pH 2. This migration diminishes with ascending pH until at pH 3 there is no cathodic migration. The isoelectric point of this preparation must therefore be in the neighborhood of pH 2.5.

Experiment 2. Isoelectric Point of Preparation III.—This experiment was carried out in exactly the same manner as Experiment 1. In Determinations 1 to 10 hydrochloric acid was used in making up the different pepsin solutions; in Nos. 11 and 12, dilute lactic acid; in Nos 13 to 17, dilute acetic acid; in Nos. 18 to 22 approximately N/10 acetic acid and sodium acetate; in Nos. 23 and 24, N/10 hydrochloric acid with sodium acetate;

TABLE III.

Preparation III. Migration of Pepsin under the Influence of an Electric Current.

Determination No.	Normality.		Digestion.		Ratio.	Duration.	pH
	Before.	After.	Cathode.	Anode.			
			mm.	mm.		hrs.	
1	0.050	0.050	1.75	2.0	0.87: 1.0	5	
2	0.050	0.050	1.25	2.0	0.62: 1.0	5	1.30*
3	0.048	0.048	2.0	2.75	0.72: 1.0	4	
4	0.048	0.048	1.5	1.5	1.0 : 1.0	4	1.31*
5	0.012		2.0	2.5	0.80: 1.0	7	
6		0.011	2.0	3.0	0.69: 1.0	8	1.96*
7	pH 2.0	pH 2.0	3.5	2.0	1.75: 1.0	4	
8	" 2.0	" 2.1	4.5	2.5	1.8 : 1.0	4	
9	" 2.0	" 2.0	2.5	1.0	2.5 : 1.0	7	
10	" 2.0	" 2.0	2.5	1.0	2.5 : 1.0	8	2.0
11	" 2.5		Trace.	Trace.	Trace.	4	
12		" 2.4	"	"	"	4	2.4
13†	" 2.7		0	4.0	0 : 4.0	4	
14		" 2.6	Trace.	Trace.	Trace.	4	
15	" 2.7	" 2.6	"	"	"	5	
16	" 2.7	" 2.6	0.5	0.5	1.0 : 1.0	6	
17	" 2.7	" 2.6	0.5	0.5	1.0 : 1.0	9	2.6
18	" 3.0	" 3.0	0	2.6	0 : 2.6	4	
19	" 3.0	" 3.0	0	1.75	0 : 1.75	5	
20	" 3.0	" 3.0	0	3.0	0 : 3.0	4	3.0
21	" 3.4	" 3.3	0.5	3.0	1 : 6.0	10	
22	" 3.4	" 3.4	0	1.7	0 : 1.7	4	3.4
23	" 5.6	" 5.6	0	1.75	0 : 1.75	4	
24	" 5.6	" 5.6	0	1.0	0 : 1.0	4	5.6
25	" 2.0	" 3.0	0	3.5	0 : 3.5	4	
26	" 2.0	" 3.0	0	3.0	0 : 3.0	5	3.0

* Calculated assuming complete ionization.

† This determination must be wrong because further determinations at the same pH give different results, so it is not considered.

The isoelectric point exhibited by Preparation III is practically identical with that shown by Preparation II. The pepsin migrated solely towards the anode at pH 3 and above, and showed maximum cathodic migration at pH 2.

and in Nos. 25 and 26, hydrochloric acid. Dilute hydrochloric acid was used in the anode electrode for Determinations 1 to 17 inclusive. This was replaced in Determinations 18 to 26 by a 0.9 per cent sodium chloride solution which was slightly acidified with hydrochloric acid. In Determinations 25 and 26 the acid concentration of the anode electrode vessel was not sufficient to replace the loss in hydrogen ion sustained, through migration, by the central and side chambers. A considerable portion of the current was therefore carried by the sodium ions, thus resulting in the migration of the sodium ions to the central and side chambers with a corresponding decrease in the hydrogen ion concentration of these solutions. The pepsin migration manifested under such a condition would be determined by the minimum hydrogen ion concentration attained. The experimental results show this to be the case (Table III).

6. (a) Effect of Pepsin on Conductivity of Hydrochloric Acid Solutions.

A 0.4 per cent pepsin solution (Preparation III) in approximately 0.2 per cent hydrochloric acid was made up and left at room temperature for 4 hours. The conductivity of the solution was then compared with that of hydrochloric acid of the same concentration.

Temperature.	Specific conductivity of HCl.	Specific conductivity of pepsin solution.
°C.	*mhos*	*mhos*
3.2	0.01491	0.01477
	0.01477	0.01477
	0.01477	0.01462
	0.01477	0.01477
Average.....	0.01480	0.01473

The solutions were tightly corked and placed in an incubator at 37°C. for 5 hours and the conductivity redetermined:

Temperature.	Specific conductivity of HCl.	Specific conductivity of pepsin solution.
°C.	*mhos*	*mhos*
3.0	0.01477	0.01456
	0.01477	0.01435
	0.01477	0.01456
	0.01491	0.01435
Average.....	0.01480	0.01445

The solutions were tightly corked and placed in an incubator at 37°C. for 44 hours:

Temperature.	Specific conductivity of HCl.	Specific conductivity of pepsin solution.
°C.	*mhos*	*mhos*
3.0	0.01477	0.01422
	0.01462	0.01384
	0.01491	0.01384
	0.01477	0.01380
Average.....	0.01477	0.01392

Decrease in specific conductivity, 0.00085 mhos.

These results show that the conductivity of hydrochloric acid is only very slightly influenced by pepsin. However, if the pepsin solution is incubated for some time there is a slight but noticeable decrease in conductivity. This decrease in conductivity probably arises from one of two causes: firstly, the hydrolysis of protein impurities present in the pepsin preparation with the consequent liberation of free amino groups, which combine with the hydrochloric acid; secondly, the hydrolysis of the pepsin molecules themselves with the liberation of free amino groups. Unpublished experiments by the author show that when pepsin solutions are kept at 37°C. for even as short a time as 24 hours there is a noticeable decrease in their proteolytic activity, so it is probable that the second explanation is the correct one.

XUM

TABLE IV.

Temperature.	E.M.F. of pepsin solution.	E.M.F. of HCl.
°C.	mv.	mv.
19	309.5	309.7
	309.5	309.7
	309.7	309.7
	309.7	309.6
Average	309.6	309.7
pH	1.038	1.040
H^+	9.16×10^{-2}	9.12×10^{-2}

TABLE V.

Determination after 6 hrs. at room temperature.			Determination after 15 hrs. at 37°C.		
Temperature.	E.M.F. of pepsin solution.	E.M.F. of HCl.	Temperature.	E.M.F. of pepsin solution.	E.M.F. of HCl.
°C	mv.	mv.	°C.	mv.	mv.
20	325.3	325.3	20	324.5	325.3
	325.2	325.3		324.5	325.3
	325.1	325.4		324.7	325.4
	325.3	325.4		324.5	325.4
	325.3			324.8	
Average	325.2	325.35		324.6	325.35
pH	1.314	1.317		1.303	1.317
H^+	4.85×10^{-2}	4.82×10^{-2}		4.98×10^{-2}	4.82×10^{-2}

TABLE VI.

Temperature.	E.M.F. of pepsin solution.	E.M.F. of HCl.
°C.	mv.	mv.
20	326.5	325.3
	326.5	325.4
	326.4	325.3
	326.4	325.4
Average	326.45	325.35
pH	1.336	1.317
H^+	4.61×10^{-2}	4.82×10^{-2}

Decrease in H^+, 1.9×10^{-3}

(b) *Effect of Pepsin on Hydrogen Ion Concentrations of Acid Solutions.*

Purified pepsin (Preparation III) was dissolved in dilute hydrochloric acid and the hydrogen ion concentration of this solution compared with that of the hydrochloric acid itself. A Leeds and Northrup hydrogen ion apparatus, with a saturated potassium chloride calomel electrode, was used in all these determinations.

Experiment 1.—0.0900 gm. of purified pepsin was dissolved in 15 cc. of acid and put aside at room temperature for about 48 hours. The results are given in Table IV.

Experiment 2.—0.2000 gm. of purified pepsin was dissolved in 50 cc. of approximately 0.2 per cent hydrochloric acid and the hydrogen ion concentration of it compared with that of the stock hydrochloric acid after 6 hours at room temperature. The results are given in Table V.

Experiment 3.—0.1000 gm. of purified pepsin was dissolved in 25 cc. of acid and the solution incubated at 37°C. for 44 hours. The experimental results, given in Table VI, show that pepsin has no noticeable influence on the hydrogen ion concentration of acid solutions. If the solutions, however, are kept at 37°C. for some time there is a small but noticeable decrease in the hydrogen ion concentration.

7. Effect of Ether Extraction on Activity of Purified Pepsin.

Experiment 1.—About 0.1 gm. of pepsin was placed in an extraction thimble and extracted with ether in a Soxhlet apparatus for 2½ hours. The activity of the residue was then compared with that of the original sample by the Mett tube method.

	Original material.	Extracted material.
Weight in 10 cc. acid	0.0075 gm.	0.0052 gm.
Digestion	5.0 mm.	4.0 mm.
Comparative digestions (calculated)	5.0 "	4.8 "

Experiment 2.—This experiment was carried out in exactly the same manner as Experiment 1, except that the extraction was continued for 5 hours.

	Original material.	Extracted material.
Weight in 10 cc. acid	0.0060 gm.	0.0075 gm.
Digestion	6.75 mm.	8.0 mm.
Comparative digestion (calculated)	7.5 "	8.0 "

The above results show that extraction with ether does not affect the proteolytic activity of pepsin.

TABLE VII.

Determination No.	Solution taken.	Alcohol added.	Ether added.	Volume after evaporation.	Digestion standard.	Digestion filtrate.	Digestion precipitate.
	cc.	*cc.*	*cc.*	*cc.*	*mm.*	*mm.*	*mm.*
1	15	10	5	22	5.0	0.5	0
2	18	9	6	20	5.4	1.5	0
3	10	10	5	15	5.0	0	0
4	20	20	10	25	5.0	0	0
5	10	10	5	20	5.0	Trace.	0
6	10	10	10	20	5.0	0	0

TABLE VIII.

Determination No.	Solution taken.	Alcohol added.	Ether added.	Volume after evaporation.	Digestion standard.	Digestion filtrate.	Digestion precipitate.	Comparative digestion.	
								Standard.	Filtrate.
	cc.	*cc.*	*cc.*	*cc.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	20	10	5	32	7.5	6.2	0	7.5	7.5
2	60	25	20	45	9.5	10.0	Trace.	9.5	8.6
3	10	10	5	17	9.5	5.0		9.5	6.5
4	10	10	10	10	10.0	8.0	8.0	10.0	8.0

8. Effect of Alcohol and Ether on Pepsin Solutions in Various Acid Concentrations.

During the adsorption experiments with cholesterol it was found that if the cholesterol-alcohol-ether solution was added to an acid solution of pepsin the enzyme was almost completely inactivated. In order to ascertain the cause of this inactivation, it was decided to determine the effect of an alcohol-ether mixture on solutions of pepsin in various acid concentrations. Experiments were therefore carried out, using hydrochloric and phosphoric acid solutions of pepsin.

Experiment 1.—Purified pepsin was dissolved in 0.2 per cent hydrochloric acid and various amounts of alcohol and ether added. If a precipitate formed it was filtered off and the filtrate placed in an evaporating dish and left until the odor of ether had disappeared. The activity of the solution was then compared with that of the original untreated pepsin solution (standard). The portion of the filter paper containing the precipitate was

TABLE IX.

Determination No.	Normality.	Solution taken.	Alcohol added.	Ether added.	Volume after evaporation.	Digestion standard.	Digestion filtrate.	Digestion precipitate.	Comparative digestion.	
									Standard.	Filtrate.
		cc.	*cc.*	*cc.*	*cc.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
1	0 685	10	10	5	14	4.7	1.0	0	4.7	1.2
2	0.685	10	10	5	7.5	4.6	1.5	0	4.6	1.3
3	0.685	10	10	10	14	4.7	0	0	4.7	0
4	0.685	10	10	10	7.5	4.7	0	0	4.7	0
5	0.685	10	15	0	16	4.7	2.7	2.5	4.7	3.4
6	0.685	10	20	0	18	4.7	1.0	1.5	4.7	1.3
7	0.342	10	10	5	10	6.0	3.5	Trace.	6.0	3.5
8	0.342	10	10	5	11	6.0	2.4	"	6.0	2.5
9	0.137	10	10	5	5	6.0	5.0	2.2	6.0	3.5
10	0.137	10	10	5	12	6.0	2.4	2.3	6.0	2.5
11	0.068	10	10	5	13.5	4.6	2.7	3.0	4.6	3.1
12	0.068	10	10	5	7.5	4.3	3.3	3.0	4.3	2.8
13	0.068	10	10	10	14	4.7	2.7	2.5	4.7	3.2
14	0.068	10	10	10	8	4.3	3.3	2.9	4.3	2.9
15	0.068	10	15	0	17	4.7	3.3	3.0	4.7	4.3
16	0.068	10	20	0	21	4.7	2.7	3.0	4.7	3.9

put into 10 cc. of 0.2 per cent hydrochloric acid, Mett tubes added, and the digestive power determined in the usual manner. The results are given in Table VII.

Experiment. 2.—Same as above except that the pepsin solutions were previously dialyzed against distilled water. pH about 3.5. Table VIII shows the results.

Experiment 3.—Purified pepsin was dissolved in phosphoric acid solutions of various concentrations and treated in the same manner as in the previous experiments. The results are given in Table IX.

These results show that the enzyme is readily destroyed at moderately high hydrogen ion concentrations by alcohol-ether mixture, but that it is not affected in solutions of low hydrogen ion concentrations. Mixtures of ether and alcohol also appear to exert a greater destructive reaction than alcohol itself.

DISCUSSION.

Adsorption of pepsin with aluminium hydroxide, dissolving the hydroxide with dilute acid, and precipitating the pepsin by dialysis gave quite an active preparation, but further attempts at its purification by solution in acid and reprecipitation by dialysis were unsuccessful, a great amount of the active principle being lost during the treatment. The safranine precipitation method, on the other hand, gave a very active preparation. This method of purification has a great advantage over all previous methods employed in the purification of pepsin, for safranine does not precipitate much, if any, of the accompanying protein impurities. This is evident from the fact that, although the active principle is quantitatively precipitated from solutions of commercial pepsin, yet the weight of the precipitate is only about 5 per cent of the material taken. The usual methods of purification by precipitation with protein precipitants such as sodium chloride, ammonium sulfate, basic and neutral lead acetate, must yield products containing considerable proteins. Even precipitation by dialysis, as carried out by Pekelharing and by the writer, apparently result in the precipitation of some of the protein impurities as well as the pepsin. This is supported by the fact that the preparations obtained during this investigation by adsorption with aluminium hydroxide and subsequent dialysis were only about 70 per cent as active as those obtained by the safranine precipitation method. A comparison of the results of the chemical analysis of these various preparations with those obtained from typical animal proteins lends additional support to the above hypothesis. The greater the degree of purification the less the analysis resembles that of typical proteins.

	Typical animal proteins.	Pekelharing's pepsin.	Preparation II.	Preparation III.
	per cent	*per cent*	*per cent*	*per cent*
C.....	50.5 -54.6	51.97	48.67	45.24
H.....	6.5 - 7.3	7.07	6.84	6.19
N.....	15.0 -17.6	14.44	13.46	11.56
S.....	0.50- 2.2	1.65	1.04	1.79
P.....	0.42- 0.85	0.01	0.27	

The isoelectric point of two pepsin preparations was determined and found to be pH 2.5. This agrees fairly well with Michaelis and Davidsohn's value of pH 2.26. These results, as well as the fact that safranine, a basic dye, quantitatively precipitates the active principle, are strong evidence in favor of the assumption that the enzyme possesses very marked acidic characteristics. The basic characteristics must consequently be very low. That this is probably so is evident from the fact that on the acid side of the isoelectric point, where basic characteristics should be most evident, there is no noticeable decrease either in the hydrogen ion concentration or in the conductivity of an acid solution after addition of purified pepsin. Further evidence also in favor of the above assumption is the low percentage of the total nitrogen present as hexone base nitrogen; that is, as arginine, histidine, and lysine nitrogen. These amino acids must be responsible for most of the basic characters of protein. Therefore, since only 7.44 per cent of the total nitrogen in Preparation II was present as hexone base nitrogen, it is evident that its basic characters must be correspondingly low.

The effect of a mixture of equal amounts of alcohol and ether on pepsin in solution was found to depend to a great extent on the hydrogen ion concentration of the solution, the enzyme being inactivated in solutions of high acidity while its activity was unaffected in solutions of low hydrogen ion concentration. The extraction of solid pepsin with ether, on the other hand, does not affect the proteolytic activity of the enzyme.

SUMMARY.

1. The adsorption of pepsin by animal charcoal, Lloyd's reagent, kaolin, salicylic acid, acetanilide, cholesterol, camphor,

aluminium oxide, and freshly precipitated aluminium hydroxide, was studied and found to vary greatly for the different adsorbents.

2. Pepsin preparations were obtained by different methods and analyzed. The most active preparation was obtained by precipitating the pepsin with safranine and removing the safranine with isoamyl alcohol. A comparison of the various analyses with those from typical proteins shows that the greater the purity of the preparation the less its composition resembles that of typical proteins.

3. Purified pepsin was hydrolyzed and a number of amino acids isolated from the hydrolytic products with infinitesimal traces of purine bases.

4. The nitrogen distribution of the products of hydrolysis was determined according to Andersen and Roed-Müller's method. In these products monoamino acid nitrogen greatly preponderated.

5. The isoelectric point of two different preparations was determined and found to be pH 2.5.

6. Determinations were carried out on the effect of pepsin on the hydrogen ion concentration and conductivity of acid solutions. The results show that purified pepsin has no immediate appreciable effect on either of the properties. However, if the solution was incubated for some time a small but definite decrease was obtained, both in conductivity and hydrogen ion concentration.

7. Various preparations of pepsin were extracted with ether in a Soxhlet apparatus. The activity of the preparation was not affected by this treatment.

8. The effect of adding mixtures of alcohol and ether to solutions of pepsin of various hydrogen ion concentrations was studied. The results showed that the enzyme was rapidly inactivated in solutions of moderately high hydrogen ion concentrations by alcohol-ether mixtures but that it was unaffected in solutions of low hydrogen ion concentrations.

In conclusion, I wish to express my thanks to the Research Council of Canada, who made this investigation possible by the grant of several studentships to the author, and to Professor A. B. Macallum, under whom this research was carried on, for his kind direction and advice.

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XUM

THE EFFECT OF POTASSIUM OXALATE ON ELECTROLYTES OF BLOOD AND PLASMA.

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The common use of potassium oxalate as an anticoagulant and the probability that its use would affect the electrolyte distribution of blood led to a comparison of oxalated and defibrinated blood to find out if the use of this anticoagulant was justifiable in studies involving accurate analysis of electrolyte equilibria in blood. While few observers have had definitely as their object such a direct comparison of oxalated and defibrinated blood, a number have, in the course of other investigations, studied the two kinds of blood with respect to certain properties. The earlier work is concerned with the demonstration that the process of defibrination does not alter the electrolyte distribution.

Fredericq in 1878 (1) found this true of bicarbonate distribution. Hamburger in 1893 (2) extended the findings to the chloride and carbonate partition, and also determined that total solid and reaction of serum and "natural plasma" of both arterial and venous horse blood were identical. Christiansen, Douglas, and Haldane (3) claim, on the basis of indirect evidence, that the partial pressure of carbon dioxide of blood directly from the blood vessel must be distinctly lower than that of the same blood after defibrination. Their statement is based on the observation of a progressive fall in the carbon dioxide-combining power of defibrinated blood when exposed for a period of time (up to 5 hours) to a temperature of 37°C. They state that this difference between living and defibrinated blood is independent of the different degree of oxygen saturation of the two kinds of blood. Zuntz, whom they quote in substantiation of their statement, considers that the difference between living venous and defibrinated blood is due, at least in part, to the relative deoxygenation of the former. Warburg (4) found chloride and bicarbonate distribution in defibrinated and in hirudinized (horse) blood identical. The bulk of evidence indicates that defibrinated blood may be considered the same as the blood in the blood vessel.

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Previous studies of the effects of potassium oxalate show no agreement among the investigators. Haggard and Henderson (5) stated that, although defibrinated blood has originally a higher carbon dioxide capacity than oxalated or untreated blood, exposure of defibrinated blood to low carbon dioxide tensions causes an irreversible loss of carbon dioxide capacity. Oxalated blood does not exhibit this decrease in power to bind carbon dioxide. This verified earlier work of Christiansen, Douglas, and Haldane (3). This was disproved by Austin, Cullen, and Peters (quoted in Peters *et al.* (6)).

In Joffe and Poulton's (7) work, the difference of carbon dioxide-combining power in oxalated and defibrinated blood, whether oxygenated or reduced, lies practically within the error of their method. But these authors show a distinctly higher carbon dioxide-combining power for oxalated plasma than for defibrinated serum. Their results, although remarkably consistent, are not decisive because they are derived from a statistical comparison of blood specimens analyzed under variable conditions and on widely separated occasions. Warburg (8) considers the addition of oxalate to blood as analogous to the addition of an equivalent amount of hypertonic saline solution. The serum of blood to which he had added hypertonic sodium chloride solution combined with slightly more carbon dioxide than the serum of blood treated with isotonic saline.

Experimental Procedure.

In the earlier studies on the effect of potassium oxalate, an attempt was made to use heparin (we were unable to secure hirudin) as an anticoagulant for comparison with potassium oxalate. We were forced to abandon the use of heparin, because the differences in oxalated and heparinized specimens were so marked that the reaction of the anticoagulants was suspected. The heparin was found to be quite acid and the potassium oxalate quite alkaline to phenol red. Recrystallization of potassium oxalate renders the salt neutral: less than 2 drops of 0.01 N NaOH are necessary to bring 10 cc. of a 1 per cent solution of the recrystallized salt to pH 7.35 to 7.4. Unfortunately the heparin could not be neutralized, so its use was replaced by defibrination of the blood. Since all the direct evidence quoted in the introduction goes to prove that electrolyte distribution is not influenced by defibrination, we feel justified in assuming that defibrinated blood is the same as the blood in the blood vessel provided the necessary precautions are observed in handling it.

Collection of Blood.—All blood was drawn from human subjects, patients on the Medical Wards of the New Haven Hospital. There

were two general types of experiments, "capacity" and "content" experiments. In "capacity" experiments, the blood was collected without precautions against contact and kept chilled until ready for use. It was then saturated with a definite tension (usually 40 mm.) of carbon dioxide in air. There were two types of "content" experiments. "Content" either means that the defibrinated blood was treated with oxalate directly after saturation with known carbon dioxide tension without exposure; or, that the blood was drawn from the subject directly into sampling tubes over mercury and defibrinated or oxalated without exposure. The latter type is a "true content" experiment.

Three different methods were used for the collection of the blood for capacity experiments:

Method A.—The total sample was withdrawn into an open vessel and defibrinated by stirring with a glass rod. Half of the defibrinated blood was added to enough solid neutral potassium oxalate to make a 0.2 per cent solution of the salt in the whole blood (Table II, Cases 12, 13, and 14).

Method B.—The blood sample was drawn from the vein through a three-way stop-cock, connected directly with the needle, alternately into two tubes, one of which contained enough neutral potassium oxalate to make an approximately 0.2 per cent solution of the salt in the blood. The stop-cock was turned every 8 or 10 seconds during the withdrawal of the blood, so as to insure original uniformity of the two samples. The blood in the tube containing no salt was defibrinated by stirring with a glass rod (Table I, except Case 9).

Method C.—The blood was withdrawn as in Method B, and then one-half of the defibrinated blood was mixed with enough potassium oxalate to make a 0.2 per cent solution. There were thus in this case three samples for comparison: oxalated blood, defibrinated blood, and defibrinated blood containing potassium oxalate (Case 9, Tables I and II).

For the content experiments blood was treated in two ways.

Method A.—A large amount of blood (usually 50 to 80 cc.) was defibrinated and then saturated at 40 mm. of carbon dioxide. Some of the saturated blood and serum separated from the blood was analyzed as defibrinated blood. The rest of the blood, after saturation, was delivered directly without air contact into a sampling bulb full of mercury and coated with enough potassium oxalate to make a 0.2 per cent solution. From a portion of this blood, serum was also separated. Whole blood and serum were analyzed as "defibrinated plus oxalate" blood. In two of the experiments, one-half of the oxalated blood was resaturated and analyzed (Table III).

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Method B.—After the new technique (described in the succeeding article) (9) for defibrinating blood out of contact with air had been devised, the blood was drawn into a syringe under oil. One-half was delivered directly into a sampling bulb and defibrinated with mercury; the other half was delivered directly into a sampling bulb containing enough potassium oxalate to make a 0.2 per cent solution in the blood (Table III, Case 18).

Preparation and Use of Potassium Oxalate.—Technical potassium oxalate is recrystallized from hot water and washed twice with cold water. The recrystallized salt should be kept in Pyrex containers. In the course of months, it gradually becomes more alkaline, so it is well to recrystallize about every 6 weeks.

The introduction of the potassium oxalate into the blood is a matter of some importance. The desired amount of the neutral salt is made up in a fresh concentrated solution and introduced into the container which is to receive the blood. The mixture is dried by drawing a current of air through the vessel; frequent rotation of the vessel during the drying insures a uniform, thin coating of oxalate. When the blood is introduced it comes into contact with only a little oxalate at a time, which is immediately dissolved. This method obviates the danger of hemolysis which results when too great a concentration of oxalate comes into contact with only a small amount of blood, insures uniform rapid equilibrium conditions, and prevents the formation of any small clots.

Analytical Procedure.

Cell volume was determined by using a Daland hematocrit fitted to a No. 1 International Equipment Company, centrifuge. Duplicate determinations were carried out on each blood sample and always agreed within 1 volume per cent.

Oxygen capacity was determined in the Van Slyke constant pressure pipette by a method devised by Lundsgaard and Neill.[1] Sometimes the blood cells from which the serum or plasma had been removed were used for oxygen capacity determinations as described in a previous communication (10).

Carbon dioxide was determined by the Van Slyke and Stadie method (11) in a calibrated constant pressure Van Slyke pipette.

Saturation of the blood with carbon dioxide was effected by the second saturation method described by Austin *et al.* (12).

Chlorides.—Until October 1, 1923, the chlorides were determined by the Austin and Van Slyke method (13) with the modification described by us elsewhere (10). Later determinations were done by the Van Slyke method (14).

Proteins.—Serum or plasma proteins were determined by macro-Kjeldahl procedure using $\frac{2}{3}$ to $\frac{1}{2}$ cc. samples.

[1] Personal communication.

Experimental Results.

Capacity Experiments.—The results of the simplest experiments on comparison of oxalated and defibrinated blood are to be found in Table I. The blood was collected as described under Method B of the capacity methods. Oxygen capacity was done on both defibrinated and oxalated blood as an assurance that the blood samples had been well mixed, but no differences between the two greater than the error of the method were ever observed. Whole blood chlorides were determined on either specimen. The rest of the blood was saturated with 40 mm. of carbon dioxide in air. In two cases the samples were saturated at both 30 and 60 mm. of carbon dioxide, in order to give carbon dioxide absorption curves. Defibrinated and oxalated saturated whole bloods were analyzed for cell volume and carbon dioxide content. "True serum" and "true plasma" were analyzed for carbon dioxide content, sodium chloride content, and in some cases for protein concentration. Cell carbon dioxide and sodium chloride were calculated from whole blood and plasma or serum values, and cell volume.

There is no absolute consistency in the results. The greatest consistency is to be found in the cell volume changes. In all but one case (probably an error), oxalate caused contraction of the cells.

The carbon dioxide of whole blood and of plasma is very little changed in average experiments. Individual samples show considerable variations in both directions. The cell bicarbonate differences are, of course, greater than those of whole blood or serum; for, if the latter values change only slightly and the cell volume varies considerably, the cells must sustain great changes.

Average values for plasma and serum sodium chloride show little change, but individual samples show the same relatively large variations as do the bicarbonate values.

In Table II are given the data which were obtained from the comparison of defibrinated blood and defibrinated blood to which oxalate had been added. The cell volume shows an even greater tendency to lowering as a result of the addition of oxalate than in the experiments where oxalation was not complicated by defibrination. The electrolyte changes are, in general, the same as those demonstrated in Table I.

TABLE I.

Comparison of Carbon Dioxide Capacity of Oxalated and Defibrinated Blood.

Case No.	Defibrinated or oxalated blood.	Cell volume.	Oxygen capacity.	Carbon dioxide content.			Sodium chloride content.			Plasma or serum proteins.	Hemolysis.	Method of treatment of blood.	Carbon dioxide tension.
				Whole blood.	Cells.	Serum or plasma.	Whole blood.	Cells.	Serum or plasma.				
		vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*	*gm. per liter*	*gm. per liter*	*gm. per liter*	*per cent*			*mm. Hg*
1	Defibrinated.	45.65	20.15	51.3	36.7	63.55	4.75	2.93	6.28		None.	Capacity, B.	40
	0.19 per cent oxalate.	43.2	20.26	51.45	29.8	63.55		2.98	6.09	6.80	"	" "	40
2	Defibrinated.	34.4	16.48	51.85	35.3	60.5	5.02	2.73	6.22		"	" "	40
	0.33 per cent oxalate.	35.5	16.63	51.75	36.2	60.3		3.04	6.11	6.85	"	" "	40
3	Defibrinated.		16.65			51.5		3.03	6.34		(?)	" "	40
	0.31 per cent oxalate.	34.05	16.53	46.15	35.4	51.65	5.15	3.03	6.24		(?)	" "	40
4	Defibrinated.	23.95	10.05	34.9	29.5	36.3	6.10	5.34	6.34	7.10	(?)	" "	40
	0.4 per cent oxalate.	20.35	10.10	35.5	28.4	37.35		4.90	6.40	7.38	(?)	" "	40
5	Defibrinated.	50.25	21.68	43.2	34.8	51.7					None.	" "	40
	0.17 per cent oxalate.	48.5	21.55	43.25	35.1	51.85					"	" "	40
6	Defibrinated.	45.3	20.55	41.75	33.0	50.75			6.16		"	" "	40
	0.2 per cent oxalate.	44.15	20.5	41.25	29.3	51.0			6.10		Moderate.	" "	40
7	Defibrinated.	40.25	16.16	48.0	37.2	55.3	4.80	3.03	5.99		Very slight.	" "	40
	0.17 per cent oxalate.	36.65	15.95	48.0	36.3	54.8		2.73	5.99	6.61	" "	" "	40
8	Defibrinated.	43.0	16.48	45.7	34.3	54.3	4.79	2.74	6.34		Slight.	" "	40
	0.17 per cent oxalale.	39.7	16.50	46.7	35.8	53.9		2.62	6.22	6.79	"	" "	40

	0.17 per cent oxalale.	39.7	16.50	46.7	35.8	53.9		2.62	6.22	6.79	"	" "		40
9	Defibrinated.	40.75	17.35	46.6	36.2	53.75	4.52	2.35	6.01		None.	Capacity, C.		40
	0.2 per cent oxalate.	38.9	17.28	44.5	32.1	52.3		2.26	5.96	6.73	"	" "		40
10	Defibrinated.	42.6	16.68	39.6	29.3	47.25	5.35				Marked.	" B.		30
	0.2 per cent oxalate.	40.6	16.60	39.7	28.5	47.2		4.40	6.00	5.55	None.	" "		30
	Defibrinated.	43.5	16.68	52.7	40.2	62.3					"	" "		60
	0.2 per cent oxalate.	39.6	16.60	52.75	39.5	61.5		5.35	5.35	5.55	Marked.	" "		60
11	Defibrinated.	41.7		45.3	31.2	54.45					Very slight.	" "		30
	0.2 per cent oxalate.	39.9		45.1	32.6	53.35					" "	" "		30
	Defibrinated.	42.05		57.25	43.9	67.0					Slight.	" "		60
	0.2 per cent oxalate.	41.0		57.3	43.7	66.85					"	" "		60
Aver-age.	Defibrinated.	41.12	17.22	46.51	35.2	54.51		3.16	6.21					
	Oxalated.	39.01	17.19	46.44	33.9	54.28		3.08	6.14					

TABLE II.

Capacity Experiments. Comparison of Defibrinated Blood with Defibrinated plus Oxalated Blood and Also with Defibrinated plus Sodium Chloride Blood.

Case No.	Defibrinated or defibrinated plus oxalated blood.	Cell volume.	Oxygen capacity.	Carbon dioxide content.			Sodium chloride content.			Serum proteins.	Degree of hemolysis.	Method of treatment of blood.	Carbon dioxide tension.
				Whole blood.	Cells.	Serum.	Whole blood.	Cells.	Serum.				
		vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*	*gm. per liter*	*gm. per liter*	*gm. per liter*	*per cent*			*mm. Hg*
12	Defibrinated.	48.0	22.48	44.55	30.3	57.75	5.22	4.19	6.17		None.	Capacity, A.	40
	" plus 0.45 per cent oxalate.	40.65	22.10	46.0	27.2	58.85		4.05	6.01	6.41	Marked.		40
9	Defibrinated.	40.75	17.35	46.6	36.2	53.75	4.52	2.35	6.01		None	Capacity, C.	40
	" plus 0.22 cent oxalate.	38.7	17.16	47.0	35.9	54.0		2.30	5.92	6.73	Slight.		40
13	Defibrinated.	46.85	21.40			50.45				7.23	Marked.	Capacity, A.	40
	" plus 0.2 per cent oxalate.	43.80	21.32			52.6				6.40	"		40
14	Defibrinated.	48.41	20.33	43.05	26.3	52.8				7.24	Moderate.	Capacity, A.	40
	" plus 0.2 per cent oxalate.	43.58		43.70	33.8	53.35				6.92	"		40
	Defibrinated plus 0.07 per cent sodium chloride.	44.73		44.25	33.4	53.0				7.04	"		40
Average.	Defibrinated.	46.00	20.41	44.73	30.9	53.69		3.27	6.09				
	" plus oxalate.	41.68	20.19	45.57	32.3	54.70		3.17	5.97				

It should be emphasized that the bicarbonate and chloride differences observed here, though slight, represent real changes. While the chloride method is less delicate than the carbon dioxide method, it is accurate to within 0.1 gm. of sodium chloride per liter. Duplicate carbon dioxide analyses, even if the duplicates are separately saturated, regularly agree within 0.2 volume per cent of carbon dioxide.

Plasma and serum proteins cannot be directly compared, because fibrin has been removed from the serum. In the only case where both plasma and serum proteins were determined the plasma value is higher than the serum value, probably because the dilution of the plasma by water derived from contraction of the cells more than compensated for the removal of the fibrin.

Content Experiments.—The results of the content experiments are given in Table III. The blood in Cases 15, 16, and 17 was treated as described under content collection Method A; that of Case 18 was collected as described under content Method B.

Considering Cases 15, 16, and 17, the cell volume shows the customary shrinkage on the addition of oxalate. The whole blood carbon dioxide content is the same in defibrinated and defibrinated plus oxalated blood. In one case (No. 16) serum carbon dioxide remains unaltered; in Cases 15 and 17 it drops in the oxalated specimen. There are two resaturation experiments, Cases 16 and 17. Although the defibrinated plus oxalated blood is resaturated at the tension used in the original saturation of the defibrinated blood, greater differences from both the defibrinated and defibrinated plus oxalated blood are observed in the resaturated sample. Even the whole blood carbon dioxide changes in Case 16. It is evident, therefore, that the addition of oxalate to blood as drawn (that is, at constant carbon dioxide content) alters the carbon dioxide tension, while oxalate added to blood at constant carbon dioxide tension, alters the carbon dioxide content.

The true content experiment (Case 18) shows about the same differences as do the others. A difference in the oxygen capacity was observed here. However, when the cells of the oxalated specimen were diluted, a viscous dark mixture resulted. Some of the hemoglobin may have been destroyed and it was difficult to measure the specimen for analysis. These considerations are probably responsible for the low oxygen capacity of the oxalated sample.

TABLE III.

Content and Resaturation Experiments.

Case No.	Defibrinated, defibrinated plus oxalated, or oxalated blood.	Cell volume.	Oxygen capacity.	Carbon dioxide content.			Sodium chloride content.			Serum proteins.	Hemolysis.	Method of treatment of blood.	Carbon dioxide tension.
				Whole blood.	Cells.	Serum or plasma.	Whole blood.	Cells.	Serum or plasma.				
		vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*	*gm. per liter*	*gm. per liter*	*gm. per liter*	*per cent*			*mm. Hg*
15	Defibrinated.	45.55	19.80	45.55	35.1	54.3				6.83	Moderate.	Content, A.	40
	" plus 0.2 per cent oxalate.	43.55	19.73	45.6	35.1	53.7				6.44	"	" "	40
16	Defibrinated.	42.4	18.05	48.15	35.6	57.3				5.38	Slight.	Content, A.	40
	" plus 0.2 per cent oxalate.	38.2		48.2	33.8	57.15					"	" "	40
	" " resaturated.	38.9		47.45	33.0	56.6					Moderate.	" "	40
17	Defibrinated.	46.0	18.89	45.4	34.2	54.9				5.26	None.	Content, A.	40
	" plus 0.2 per cent oxalate.	41.1		45.3	32.9	53.95					"	" "	40
	" " resaturated.	43.4		45.2	34.9	53.05					Very slight.	" "	40

Average.	Defibrinated.	44.65		46.37	35.0	55.5							
	" plus oxalate.	40.95		46.37	33.9	54.93							
18 True content experiment.	Defibrinated.	51.7	21.36	46.65	36.9	57.15	4.28	2.78	5.87		None.	Content, B.	40
	0.2 per cent oxalate.	45.9	20.47	46.55	35.0	56.3	4.34	2.70	5.73		Very slight.	" "	40

Comparison of Addition of Osmotically Equivalent Amounts of Sodium Chloride and Potassium Oxalate to Defibrinated Blood.—Enough oxalate was added to defibrinated blood (Case 14, Table II) to make a 0.2 per cent solution. Enough sodium chloride was added to some of the same defibrinated blood to make a solution of sodium chloride in blood osmotically equivalent to a 0.2 per cent solution of oxalate.

The cell shrinkage is less, while the change in the whole blood bicarbonate is greater in the sodium chloride specimen; cell and serum bicarbonate are much the same in the chloride and oxalate samples, being greater in both than in defibrinated blood. The changes in serum protein concentration and cell volume in the three specimens are closely correlated.

Rate of Sedimentation of Cells in Oxalated and Defibrinated Blood.—Blood containing 0.2 per cent potassium oxalate and a sample of the same blood defibrinated were placed in narrow tubes and the time rate of settling of the cells was determined. The cells of the oxalated sample settled more rapidly. At the end of 2 hours the serum volume of the defibrinated specimen was 0.02 cc., that of the oxalated 0.18 cc. Complete sedimentation in the oxalated specimen was reached in 8 hours; complete sedimentation of cells in the defibrinated specimen was not attained at the end of 50 hours. The experiment was repeated, using for the comparison defibrinated blood and defibrinated blood containing oxalate. The serum volume of the two specimens after 2 hours, was approximately the same.

DISCUSSION OF RESULTS.

There seems to be no doubt that oxalate, in concentrations usually employed to prevent coagulation of blood, causes the blood cells to contract. The cell volume shrinkage is not regularly proportional to the oxalate concentration, although the average shrinkage is greater in those experiments in which high concentrations of oxalate were used. In nine specimens where the oxalate concentration is 0.17 to 0.2 per cent, the cell volume of the oxalated specimen varies from that of the defibrinated specimen by 1.15 to 3.60 volumes per cent, an absolute cell shrinkage of 2.5 to 9.0 per cent. However, if the concentration of oxalate is very excessive (0.4 to 0.6 per cent), there is a relatively larger absolute

shrinkage (14 to 15 per cent). It may be pointed out that no direct relationship is to be expected between cell volume changes and oxalate concentration in different bloods because of the effect of other variables; the direct relationship would presumably appear in comparisons on one sample of blood. Both the concentration of carbon dioxide and chlorides and their distribution also may be slightly but significantly altered by the addition of oxalate, but neither the magnitude nor direction of these changes in a given specimen of blood can be predicted.

Contraction of the blood cells under the influence of oxalate is what one would expect if the cells are impermeable to both potassium and oxalate ions, or unequally permeable to them. It has never been determined whether the oxalate ion penetrates the cell membrane. Information on other anions has, however, been secured. De Boer (15) showed that addition of carbon dioxide to blood caused a shift of sulfate ion across the cell membrane. Hamburger (16) and Van Slyke and Cullen (17) showed that transfer of chlorine from plasma to cells results if blood is treated with carbon dioxide. These results were confirmed qualitatively but not quantitatively by Fridericia (18). Buell (19) claims that inorganic phosphate is confined to the serum, which would indicate that the cell envelopes are impervious to phosphate. Buell's observations have not been confirmed by other investigators. Rossdale (20), who limited his observations to the relative effects of citrate and oxalate on cell volume, found oxalate to have a greater effect than citrate in reducing cell volume. His results are, however, far from conclusive, since he did not weigh his salts.

The balance of evidence is against the permeability of the red cell membrane to cations. Gürber in 1895 (21) showed that alterations of carbon dioxide tension caused no transfer of sodium and potassium between cells and serum. This fact was subsequently verified by Hamburger. The latter later found that the addition of salts or water to blood did cause such a transfer (22). Recent unpublished studies from this laboratory (23) indicate that there is no transfer of sodium and potassium ions across the red cell membrane in response to additions of salts. The absence of calcium from the cells, pointed out by Halverson, Mohler, and Bergeim (24) and by Falta and Richter-Quittner (25) argues the impermeability of the membrane to calcium.

Ege (26) found that the rate of penetration of electrolytes into cells varies, and was not able to attain equilibrium short of hours with some salts. In the case of the experiments recorded here, time as a factor has been ruled out. Saturation of oxalated blood immediately after its withdrawal from the subject and saturation of a duplicate sample of the same oxalated blood 2 hours later gave identical values.

Blood Saturated.

	Immediately. *vols. per cent*	After 2 hrs. *vols. per cent*
Hematocrit	45.05	45.1
Oxygen capacity	20.8	20.9
Whole blood CO_2 capacity at 40 mm	45.65	45.6
True plasma " " " 40 "	54.15	54.3

Earlier experiments reported from this laboratory (6) showed that oxalated blood, if kept cold, would not change its carbon dioxide-combining power for at least $3\frac{1}{2}$ hours.

Record was kept of the apparent amount of hemolysis in each blood specimen. Degree of hemolysis does not seem to be correlated with the observed changes.

According to Cullen (27) and Hastings and Sendroy (28), the pH of serum and plasma is the same. It is hard to understand how this can be possible if cell volume and electrolyte distribution are as much affected as our experiments indicate. It seems more likely that the methods used by these observers were not sufficiently delicate to permit the detection of these differences. Colorimetric methods are accurate to only 0.02 pH, which corresponds to almost 2 volumes per cent of carbon dioxide on the average absorption curve. By the methods employed in our experiments changes of carbon dioxide of as little as 0.2 volume per cent can be detected with reasonable certainty.

In Cases 15, 16, and 17, we have, as in the later capacity experiments, the combined effects of defibrination and oxalate. The cell volume shrinkage is about the same as in the similar capacity experiments. As is to be expected, since the transfer of defibrinated blood to the oxalated chamber is accomplished anaerobically, the whole blood carbon dioxide content is not altered in the process. The drop in serum carbon dioxide content observed in two of the cases is to be explained, partly on the basis

of the dilution of the plasma with contraction of the cells, partly on the basis of ionic transfers across the cell membrane under the influence of potassium and oxalate ions.

It is clear then that the addition of oxalate to blood at a constant carbon dioxide tension (capacity experiments) alters the carbon dioxide content; while the same addition at constant carbon dioxide content (content experiments) alters the tension. This is emphasized in the resaturation experiment (Case 16, Table III) where both content and tension are independently varied, each variation causing distinct changes.

In Case 18 we have only the effects of potassium oxalate without the complication of defibrination. The blood here, although compared directly as drawn from the subject, without exposure to air and without resaturation with carbon dioxide, exhibits the same variations as the capacity experiments, and offers additional evidence that oxalate *per se* changes the carbon dioxide tension of the blood.

As pointed out above, Warburg claims that the action of potassium oxalate is similar to that of an equivalent amount of sodium chloride. Our failure to get comparable results from the addition of apparently osmotically equivalent amounts of potassium oxalate and sodium chloride may have been due to the fact that the amounts added were not isotonic. Sodium chloride and potassium oxalate are salts whose anions have different valencies, and their osmotic effects probably do not correspond with their ionic concentrations. When potassium oxalate is added to blood, some of the oxalate anion is precipitated by calcium, the calcium being replaced in its combinations by potassium. The calcium is confined to the serum, where there is always an excess of anion available. If calcium oxalate precipitation is taken into account in the calculations, the effect of the oxalate on cell volume and ionic distribution becomes no more consistent or predictable.

In two experiments (Table I, Cases 10 and 11) the carbon dioxide absorption curves of oxalated and defibrinated blood were compared. In both cases the whole blood carbon dioxide at 30 and at 60 mm. was the same in the oxalated and the defibrinated specimen. In Case 10 the plasma and serum carbon dioxide at 30 mm. were the same, but the 60 mm. serum was higher than the 60 mm. plasma. In Case 11 the 30 mm. serum was higher than

the 30 mm. plasma, and at 60 mm. plasma and serum were the same. If the plasma and serum curves are plotted on logarithmic paper (the logarithmic carbon dioxide absorption curve is a straight line (6)) it will be seen that they intersect (Fig. 1). In Case 10 they intersect at the 30 mm. point; in Case 11, they intersect at the 60 mm. point. The intersections in the processes occur in opposite directions. In Case 10 the serum curve is higher than the plasma at high carbon dioxide tensions; in Case 11 the serum curve is higher than the plasma at low tensions. If these were cases where defibrinated and oxalated whole blood differed

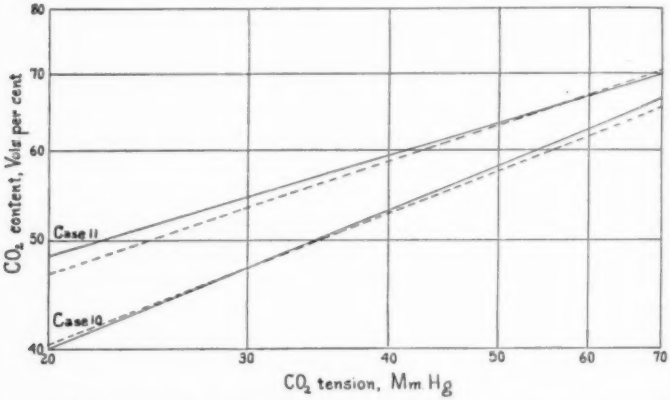

FIG. 1. Comparison of the CO_2 absorption curves of serum and oxalated plasma (Cases 10 and 11). — = serum; ---- = plasma.

at constant carbon dioxide tension, similar intersections of whole blood curves might be demonstrated. In other words, oxalate may influence both the height and the shape of the absorption curve. Such changes in the absorption curve under the influence of oxalate probably are the causes of the apparent inconsistency of the electrolyte disturbances. The effects of oxalate on bicarbonate and chloride distribution depend on whether the blood was studied at a carbon dioxide tension above or below the point of intersection of the defibrinated and oxalated absorption curves.

The rapid settling of blood cells in oxalated blood must be due to the removal of fibrin and not to the presence of oxalate, since

defibrinated blood to which oxalate has been added has the same cell sedimentation rate as defibrinated blood. This is an indication that oxalate definitely alters the physical properties of the blood. That the cell volume changes after oxalate are not due merely to physical alterations of this nature, which facilitate packing of the cells, is proved by the protein determinations in the oxalate-chloride comparison experiment. The serum and plasma protein values show evidence of a transfer of water from the cells that is in keeping with the cell volume findings. That this is true of electrolyte changes also is apparent from Experiment 18, in which the distribution of carbon dioxide in oxalated blood and defibrinated blood analyzed just as they were drawn, without air contact or saturation, differed.

CONCLUSIONS.

Since potassium oxalate has such varied and inconsistent influence on the electrolyte distribution in blood, it is impossible even to establish an average correction for its effects. Where only approximate accuracy is desired (*i.e.* carbon dioxide values within 1 volume per cent, sodium chloride to within 0.2 gm. per liter, and cell volume to within 3 volumes per cent), the use of a concentration of oxalate not exceeding 0.2 per cent may be justified, because of the fact that it affords a simple means of preventing clotting. However, where the absolute accuracy attainable by the methods used for the determination of these constituents is desired, the use of potassium oxalate as an anticoagulant is not advisable and should be replaced by defibrination.

Although this study has not proved that defibrination itself is without effect on cell volume and electrolyte distribution, the bulk of evidence would indicate that this is the case.

The fact that the cells of defibrinated blood settle very slowly is an additional reason for the use of defibrinated blood. In certain pathological bloods, if oxalated specimens are used for study, the cells settle out so rapidly that it is almost impossible to obtain a good plasma-cell mixture and it is therefore difficult to get good check duplicate whole blood analyses.

The common use of sodium fluoride, which presumably acts like sodium chloride, is also to be deplored, because it affects the electrolyte distribution and because it is unnecessary.

SUMMARY.

1. Defibrinated and oxalated blood saturated with 40 mm. of carbon dioxide in air showed no consistent differences except as regards hematocrit, which was always lower in the oxalated specimen. The variations in electrolyte (bicarbonate and chloride) distribution were not consistent in direction or degree.

2. Defibrinated blood and defibrinated blood containing oxalate showed the same consistency as regards cell volume variations and the same inconsistencies as regards variations in the electrolyte distributions as did defibrinated and oxalated blood.

3. Blood drawn and defibrinated out of contact with air, when directly compared with blood drawn onto 0.2 per cent potassium oxalate out of contact with air, showed that oxalated and defibrinated blood as drawn directly from the body are no more similar than such bloods are after exposure to a new definite tension of carbon dioxide.

4. The carbon dioxide absorption curves of oxalated and defibrinated blood are not identical.

5. The action of a given concentration of oxalate is not the same as that of an equivalent amount of sodium chloride.

6. Time is not a factor. Equilibrium conditions are the same immediately after the addition of oxalate and 2 hours after its addition.

7. The use of defibrinated blood is advised for studies involving electrolyte distribution, because the effects of potassium oxalate are unpredictable.

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XUM

METHOD FOR ANAEROBIC DEFIBRINATION OF BLOOD.

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It was pointed out in the preceding paper (1) that the electrolyte equilibrium of blood is disturbed if potassium oxalate is used as an anticoagulant and that therefore defibrination should replace the use of potassium oxalate where precise results are desired. Since no satisfactory method existed for defibrination of blood out of contact with air, it became necessary to devise a simple technique for this purpose.

Blood must be collected and defibrinated anaerobically for experiments in which the electrolyte equilibrium as it exists in the circulating blood is studied, so called "content" experiments, as distinguished from "capacity" experiments, in which the blood is drawn from the subject without precautions against exposure and is subsequently brought into equilibrium with a definite tension of carbon dioxide. For the latter type of experiment, the blood may, of course, be collected in an open tube and defibrinated in the conventional manner by stirring with a glass rod. It was particularly for content experiments that the method described here was developed.

Anaerobic defibrination of blood cannot be successfully accomplished by merely stirring oil-covered blood with a glass rod. The clot cannot be completely removed in this manner; furthermore, defibrination may require 10 to 15 minutes, which is a sufficiently long time to permit a significant gas exchange (through oil) between the blood and the outside atmosphere.

The following is a convenient and satisfactory method for defibrination of blood without exposure to air. The receptacles for the blood are blood-sampling bulbs of varying capacities (8 to 30 cc.) of Pyrex glass, similar to those described by Austin

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et al. (2). The tubes connecting the bulb with the stop-cock and the rubber tubing must be at least 3 mm. inside bore to prevent the blood from sticking in the ends.

The blood is drawn into a Luer syringe sealed with albolene and transferred immediately to the blood-sampling bulb which is filled with mercury. All but 1 or 2 cc. of mercury, the amount depending on the size of the sampling bulb, is displaced from the bulb with blood. This proportion of blood to mercury must be approximately adhered to, because less mercury will not be sufficient to defibrinate the blood, and a greater proportion of mercury tends to cause hemolysis. The sampling bulb containing blood and mercury is inverted repeatedly, care being taken that the mercury is driven into the narrow ends of the bulb with each inversion. The residual mercury acts as a plunger and defibrinater; the fibrin collects on fine globules of mercury. It is important to continue the shaking for 5 minutes after clotting has begun. The commencement of clotting is evidenced by a greater viscosity of the contents of the sampling bulb and a tendency of the blood to stick in the narrow ends of the tube.

TABLE I.

Comparison of Oxygen Capacities of Blood Aerobically and Anaerobically Defibrinated, and Oxalated Blood.

Case No.	Treatment of blood.		
	Old aerobic defibrination. Oxygen capacity.	New anaerobic defibrination. Oxygen capacity.	Oxalated. Oxygen capacity.
	vols. per cent	*vols. per cent*	*vols. per cent*
1	17.36	17.23	
2	18.87	18.73	18.68
3	16.75	16.52	

After defibrination is complete, the leveling bulb connected with the sampling tube is raised and the lower stop-cock of the tube is opened so that the blood will be under positive pressure. This is a necessary precaution, because otherwise, as the blood cools down to room temperature, gas will be evacuated from it. The clot adheres firmly to the mercury globules and sinks to the bottom of the tube, so that it never interferes with the withdrawal (under positive pressure) of the blood samples for analysis.

In order to see if any excess of cells was occluded in this method of defibrination, the oxygen capacity of blood so defibrinated was compared with that of blood defibrinated in the ordinary manner by stirring in an open tube with a glass rod. In one case it was also compared with the oxygen capacity of the same blood oxalated. (Potassium oxalate does not change the oxygen capacity of blood.) The checks were satisfactory (Table I).

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A GASOMETRIC METHOD FOR THE DETERMINATION OF pH IN BLOOD.

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There are three possible techniques by which the pH of body fluids may be determined: (1) the electrometric technique, using the hydrogen electrode, (2) the colorimetric technique, using dye or buffer standards, and (3) the gasometric technique in which the pH is determined by carbon dioxide analyses. The method described in this paper is a gasometric method, which utilizes the carbon dioxide absorption curve of "separated" serum.

The electrometric technique, the standard method to which all others must be referred, is complicated, requires expert manipulation and almost constant standardization. Colorimetric methods are simple in execution but of doubtful accuracy and limited applicability. The gasometric method thus becomes the method of choice where accuracy and wide clinical application are desiderata and where only ordinary laboratory facilities are available.

The two most widely employed colorimetric pH methods are those of Cullen (1) and of Hastings and Sendroy (2). Cullen's method employs a series of phosphate buffer mixtures as standards, comparing with these standards plasma diluted twenty-fold with normal saline which has previously been adjusted to pH 7.35 to 7.4. The diluted plasma is brought to 20°C. before comparison with the standard. Correction is made for protein, salt, and temperature effects by subtracting from the reading a standard figure, called by Austin, Stadie, and Robinson (3) the C correction. Cullen admits that the difference between colorimetric and electrometric pH readings may be altered, especially in pathological

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cases. Hence the method is of limited applicability where a variety of pathological material is studied.

Hastings and Sendroy (2) use a set of bicolor standards made of acid and alkaline mixtures of the dye, phenol red. The diluted serum and dye standards are adjusted to 38°C. This method therefore eliminates temperature effects; it does not, however, according to Austin, Stadie, and Robinson, adequately control salt and protein effects. These authors show that the errors due to such effects in colorimetric methods may cause an error of as much as 0.08 pH. Quite often when studies of pH are made it is also desirable to know the carbon dioxide tension of the serum. An error of 0.08 pH involves great inaccuracy in the determination of this value.

Austin, Stadie, and Robinson (3) have developed a method which is a combination of colorimetric and gasometric technique. This method is more complicated than those of Cullen and of Hastings and Sendroy, but it does take care of Cullen's C correction.

A less complicated gasometric technique has been developed by us, which is independent of the C correction and also serves to determine carbon dioxide tension accurately. The advantage of using a gasometric technique for pH determinations when the other determinations in a series are estimated gasometrically is the elimination of extra apparatus, colorimetric standards, or the hydrogen electrode.

Procedure.

The method here described is based on the Henderson-Hasselbalch equation (4):

$$\text{(1)} \qquad \text{pH} = \text{pK}_1 + \log \frac{\text{BHCO}_3}{\text{H}_2\text{CO}_3}$$

When $BHCO_3$ is estimated from gasometric determination of the total CO_2 content of the serum, it is calculated, in terms of volumes per cent CO_2, as:

$$\text{BHCO}_3 = \text{CO}_2 - \text{H}_2\text{CO}_3$$

$$\text{H}_2\text{CO}_3 = \frac{100\ \alpha\ \text{p}}{760}$$

where α is the solubility coefficient of CO_2 in serum at 38° and p is the CO_2 tension. Hence, when the experimentally determined data, *viz.* CO_2 content, and CO_2 tension, p, are introduced into Equation 1 it becomes

$$pH = pK_1 + \log \frac{CO_2 - \frac{100\ \alpha\ p}{760}}{\frac{100\ \alpha\ p}{760}} \qquad (2)$$

pK_1 is not a constant for whole blood: it is different for cells and plasma, and therefore varies in whole blood with the cell volume (5) as well as with the pH and the state of oxygenation (6, 7). For serum and plasma (with minimum oxalation) pK_1 is approximately constant. Using Bohr's value for α, *viz.* 0.541 for serum, the average value of pK_1 calculated from Cullen's (1) CO_2 tension, CO_2 content, and electrometric pH value in ten plasmas and six sera is 6.14. This value of $pK_1 = 6.14$ was used by Austin *et al.* (8) and was employed by Peters (9) in the construction of the logarithmic chart of the relationships of carbon dioxide tension, carbon dioxide content, and pH of serum and plasma. However, thirteen determinations on horse serum by Van Slyke, Hastings, Murray, and Sendroy (10), and a larger series on normal and pathological human sera (private communication) have established an average constant pK_1 value of 6.12. This is considered to be the most accurate series of determinations of the value of pK_1, and we have therefore employed it in the calculation described in this paper.

In order to calculate pH by the Henderson-Hasselbalch equation as expressed in Equation 2, it is obviously necessary to determine the CO_2 tension p. For this purpose the carbon dioxide absorption curve of separated serum or plasma may be used. Peters, Eisenman, and Bulger (11) and Peters (9) have demonstrated that the logarithmic CO_2 absorption curves of whole blood, true plasma, or separated plasma are straight lines. The absorption curves of whole blood or true serum cannot be used, because they are altered by changes in the state of oxygenation of the blood. Joffe and Poulton (12) pointed out that the absorption curve of plasma separated from the blood without air contact ("separated" plasma) intersects the curve of plasma separated from blood after saturation with carbon dioxide ("true" plasma) at the carbon dioxide tension at which the separated plasma was removed from the cells. pK_1 of separated plasma is constant and independent of the state of oxygenation of the blood. Separated plasma can, therefore, be conveniently used for the determination of pCO_2.

Briefly the method consists in determining the carbon dioxide content of the serum separated directly (without air contact) from the cells as drawn, saturating the separated serum at two known points of carbon dioxide tension, preferably above and

below the carbon dioxide tension of the unknown blood specimen, and analyzing the saturated serum samples for carbon dioxide. For carbon dioxide determinations the constant pressure apparatus

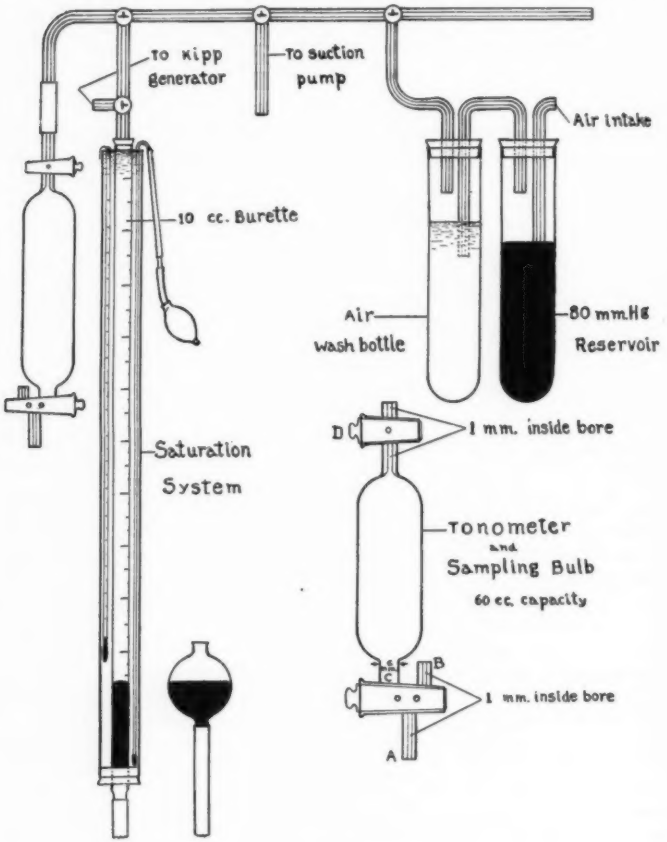

FIG. 1.

of Van Slyke and Stadie (13) with a water-jacketed burette calibrated in 0.01 cc. and the constant volume apparatus of Van Slyke and Neill (14) were employed.

Saturation is effected by the second saturation technique described by Austin *et al.* (8) at 38°C. In this method, the blood or plasma is exposed to a definite tension of carbon dioxide made up from a gas manifold. The gas in the tonometer then need not be analyzed. The ordinary macro methods of saturation in a 750 cc. tonometer are wasteful, so saturation is carried out in a small tonometer of about 60 cc. capacity, filled with gas from a calibrated fine bore 10 cc. burette, graduated in 0.05 cc. Carbon dioxide can be measured to 0.01 cc. with a probable accuracy of 0.02 cc. A sketch of the saturation system is given in Fig. 1.

The small tonometers also serve, without transfer of the serum after saturation, as sampling bulbs. One end (*A*) is equipped with a three-way stop-cock of capillary bore, one opening (*AC*) leading to the bulb and the other (*AB*) communicating with the outside air. After saturation the rubber pressure tubing joined to the leveling bulb is connected with the three-way stop-cock of the tonometer, which has been wiped dry. The air in the capillary orifice of the stop-cock is displaced by wasting mercury through the open end (*AB*). Then the mercury is allowed to rise into the sampling bulb through (*AC*) until all the gas is displaced. This process takes but a short time. After complete gas displacement, the upper two-way stop-cock (*D*) of the tonometer is shut and the lower stop-cock (*AC*) is allowed to remain open so that, as the serum cools from 38°C. to room temperature, no gas will be evacuated from the blood.

Duplicate determinations on a single point require only 3 cc. of serum.

Controls.—That such a saturation procedure checks excellently with saturation in the larger 750 to 780 cc. tonometer and that equilibrium in the large tonometers gives correct saturation values, was established by a series of controls. The controls were carried out as follows:

1. To show that a gas mixture of known CO_2 tension can be accurately prepared in small tonometers. The carbon dioxide content of serum saturated in a small tonometer with 40 mm. of carbon dioxide in air from the small manifold was compared with that of serum saturated in a large tonometer with 40 mm. of carbon dioxide in air from a large manifold. In one case (A) these were also compared with the carbon dioxide content of serum

saturated in a large tonometer with 40 mm. of carbon dioxide in air from the small manifold. The concordance in values is extremely satisfactory (Table I). It has already been shown (5) that gas mixtures can be accurately prepared in the large tonometers.

2. To show that the large system (750 cc. tonometers and 50 cc. gas burettes in manifold) permits saturation values capable of duplication. (*a*) Whole blood was saturated with 30 and 60 mm. of carbon dioxide in air by means of the large equilibrium system. The carbon dioxide capacity of whole blood and true serum was determined. A duplicate sample of blood was subjected to the same technique. The values obtained are duplicates within the error of the method (Table II). (*b*) Whole blood was saturated at either 30 or 60 mm. of carbon dioxide in air, and the plasma separated. Another portion of the separated serum was then saturated at the other tension (that is the serum separated at 30 mm. was saturated at 60 mm. and *vice versa*). Resaturation of the separated serum or duplicate saturations were made. From Table III it can be seen

TABLE I.

Comparison of Large and Small Equilibrium Systems.

Sample.	Separated serum carbon dioxide content at 40 mm. carbon dioxide tension.		
	Large tonometer, large manifold.	Large tonometer, small manifold.	Small tonometer, small manifold.
	vols. per cent	*vols. per cent*	*vols. per cent*
A	56.55	56.5	56.5
B	45.35		45.15
C	59.4		59.4

TABLE II.

Duplicate Absorption Curve of Oxalated Blood.

Saturation tension.	Carbon dioxide capacity of whole blood. Duplicates.		Carbon dioxide of plasma. Duplicates.	
	A	B	A	B
mm. Hg	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*
30	40.1	40.1	49.7	49.6
60	53.8	53.8	62.8	62.9

that the carbon dioxide content of the resaturated or separately saturated serum is the same as the original.

Aside from the experimental proof that resaturation values check first saturation values, it can be shown mathematically that the error involved in saturating only once is small. It will

TABLE III.

Duplicate Saturations or Resaturations of Separated Plasma.

	Carbon dioxide content of separated plasma at:					
	30 mm. CO_2 tension.			60 mm. CO_2 tension.		
	First saturation.	Duplicate of first saturation.	Resaturation.	First saturation.	Duplicate of first saturation.	Resaturation.
	vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*	*vols. per cent*
A. True plasma saturated at 30 mm.	49.8		49.2			
" Same true plasma saturated at 30 mm., then separated and saturated at 60 mm.				54.5	54.3	54.5
B. True plasma saturated at 60 mm.				74.8	75.2	
" Same true plasma saturated at 60 mm., then separated and saturated at 30 mm.	69.5	69.6				
C. True plasma saturated at 30 mm.	42.9		42.75			
" Same true plasma saturated at 30 mm., then separated and saturated at 60 mm.				49.5	49.45	
D. True plasma saturated at 60 mm.				33.3		33.5
" Same true plasma saturated at 60 mm., then separated and saturated at 30 mm.	27.6		27.85			

be demonstrated below that the average difference of separated serum between 30 and 60 mm. is 5.6 volumes per cent of carbon dioxide.

Then 1 mm. CO_2 tension causes a change of

$$\frac{5.6}{30} = 0.19 \text{ volumes per cent } CO_2$$

If in a 60 cc. tonometer containing 3 cc. of serum, the CO_2 tension is altered from 30 to 60 mm., the maximum change in tension produced by the CO_2 given off or taken up by the blood would be

$$\frac{3}{60} \times 30 = 1.5 \text{ mm. } CO_2$$

The effect on CO_2 content would be

$$1.5 \times 0.19 = 0.29 \text{ volumes per cent } CO_2$$

Similarly, in the large tonometer of 780 cc. containing a 6 cc. sample, the maximum change in CO_2 tension would be

$$\frac{6}{780} \times 30 = 0.23 \text{ mm.}$$

The effect on CO_2 content would be

$$0.23 \times 0.19 = 0.04 \text{ volumes per cent } CO_2$$

If, then, the saturation is carried out at a carbon dioxide tension differing by as much as 30 mm. from that of the original blood, the error involved in the determination of carbon dioxide would not exceed 0.2 to 0.3 volumes per cent. It has already been demonstrated that the error in the procedure itself is not greater than 0.2 volumes per cent. The maximum error in the whole determination should not then exceed 0.4 volumes per cent, which is equivalent to approximately $\frac{0.4}{5.6} \times 30 = 2$ mm. of carbon dioxide tension. This represents about 0.02 to 0.03 of pH.

Method in Detail.—To carry out a gasometric pH determination, the carbon dioxide content of the serum separated from the cells without air contact is determined. One sample of the separated serum is saturated at a tension below, and the other at a tension above that of the original blood. Unless the carbon dioxide content is abnormally low, 30 and 60 mm. tensions may be employed. The saturated sera are analyzed for carbon dioxide content. By means of a logarithmic pH chart (9), a straight line is drawn through the two determined points. The carbon dioxide content of the original blood serum is placed on this line

and its tension and pH can be read with considerable accuracy. Greater accuracy is obtained by calculating pCO_2 mathematically by the following formula derived from the straight line logarithmic relation between CO_2 content and CO_2 tension.

$$\log p_x = \log p_A \frac{[\log C_A - \log C_x][\log p_A - \log p_B]}{[\log C_A - \log C_B]}$$

where C = CO_2 content in volumes per cent. p = tension of CO_2 in mm. of mercury. Subscripts A and B refer to the blood samples saturated at known CO_2 tensions, x to the sample analyzed without saturation.

pH can then be calculated by means of Equation 2, Bohr's value 0.541 being substituted for α and p_x for p.

Alternative Method.—Relatively large amounts of serum are required for the method just described, but it is possible with greater error, to determine pCO_2 and pH from the carbon dioxide content of the serum separated directly from the cells without exposure to air and of the separated serum after saturation at *one* known tension. We have determined the average difference between the carbon dioxide contents of separated serum samples saturated at 30 and 60 mm. of carbon dioxide in air ($\Delta CO_{2_{60-30}}$) in forty samples of human serum, normal and pathological. Some of the values were obtained by direct analysis of the carbon dioxide content of the sera at 30 and 60 mm. Where points other than these were used, the carbon dioxide content at 30 and 60 mm. was calculated by the straight line logarithmic formula (15). The average value for ($\Delta CO_{2_{60-30}}$) is 5.63 volumes per cent of carbon dioxide, with variations from 2.9 to 9.0 volumes per cent. Thirty-five of the readings fall between 4.0 and 7.0. The lower values were obtained from two determinations on one pathological case, the upper values from a normal person who had received bicarbonate.

In the course of another investigation (16), seventeen determinations of ($\Delta CO_{2_{60-30}}$) on cat's blood were made. The values for ($\Delta CO_{2_{60-30}}$) show rather more scattering than was found in human sera, ranging from 3.0 to 7.4 volumes per cent, but the average value, 5.4 volumes per cent, is remarkably close to that of separated human serum. Although from a comparison of only

two species it is impossible to establish the fact that all mammalian separated serum slopes are practically alike within the limited range of 30 to 60 mm., still this value may prove to be more constant from species to species and from serum to plasma than is true of Cullen's C correction, which varied rather widely in different species and in serum and plasma of the same species.

This approximate value of 5.6 volumes per cent obtained from human separated sera is used to determine pH where only one saturation point and the carbon dioxide content of the unknown serum are determined. On the logarithmic pH chart a line is drawn through the determined point with a slope such that the intersections of this line with the 30 mm. line is 5.6 mm. lower than the intersection with the 60 mm. line. The unknown point is interpolated on the curve when pCO_2 and pH can be read off.

The relative accuracy of the method obviously depends somewhat on the distance between the unknown and the determined points. Table IV will illustrate this point. In making the table, the carbon dioxide content of the serum separated from the cells without air contact was used and one of the two known points was considered as the determined point. The value of the other known was calculated by using the average value of 5.6 volumes per cent ($\Delta\ CO_{2_{e-w}}$). The calculated carbon dioxide content and pH are compared with the observed content and pH. The figures are arranged so that the differences between determined and unknown points are in ascending order. There is a general tendency for the error to increase proportionately to the difference between determined and unknown points. If the difference between the two is small, the pH deviation lies within the error of the method.

This will serve to emphasize the necessity for keeping the serum out of air contact until the entire procedure is carried out. Significant loss of carbon dioxide renders more inaccurate the saturation of the specimen at a carbon dioxide tension near its original body tension.

The larger differences between determined and unknown indicated in Table IV are, of course, greater than are necessary in actually employing the one point method, because fairly close approximation can be secured if the carbon dioxide content of the

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serum separated from the cells without air contact is first determined. Fig. 2 can then be used to find approximately the tension of the original blood serum, and the separated serum can be

TABLE IV.

Error in One Point Method.

Carbon dioxide tension.					pH		
At which plasma was saturated.	Of blood as determined by two point technique.	Difference between (1) and (2).	Calculated by one point.	Error in one point method. (4) − (2).	Observed by two point method.	Calculated by one point method.	Error of one point method. (7) − (6).
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
mm. Hg	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*			
60	59.4	0.6	59.4	0	7.28	7.28	0
30	34.7	4.7	35.3	+0.6	7.44	7.43	−0.01
60	55.3	4.7	56.9	+1.6	7.31	7.29	−0.02
60	55.2	4.8	56.2	+1.0	7.27	7.26	−0.01
30	39.3	9.3	34.8	−4.5	7.44	7.49	+0.05
20	30.3	10.3	32.0	+1.7	7.39	7.36	−0.03
30	42.1	12.1	51.4	+9.3	7.41	7.32	−0.09
20	32.8	12.8	31.0	−1.8	7.40	7.43	+0.03
30	42.9	12.9	42.2	−0.7	7.45	7.46	+0.01
30	43.0	13.0	44.2	+1.2	7.39	7.38	−0.01
30	43.4	13.4	48.0	+4.6	7.40	7.35	−0.05
60	45.8	14.2	40.7	−5.1	7.42	7.47	+0.05
60	74.8	14.8	73.6	−1.2	7.15	7.16	+0.01
30	45.8	15.8	54.2	+8.4	7.42	7.34	−0.08
60	43.4	16.6	39.2	−4.2	7.40	7.45	+0.05
60	43.0	17.0	41.8	−1.2	7.39	7.40	+0.01
60	42.9	17.1	43.5	+0.6	7.45	7.44	−0.01
60	42.1	17.9	33.7	−8.4	7.41	7.51	+0.10
60	39.3	20.7	48.1	+8.8	7.44	7.34	−0.10
30	55.2	25.2	58.8	+3.6	7.27	7.24	−0.03
30	55.3	25.3	59.7	+4.4	7.31	7.27	−0.04
60	34.7	25.3	32.6	+2.1	7.44	7.46	+0.02
60	32.8	27.2	33.0	+0.2	7.40	7.40	0
30	59.4	29.4	65.7	+6.3	7.28	7.24	−0.04
60	50.3	29.7	28.3	−2.0	7.39	7.42	+0.03
30	74.8	44.8	70.4	−4.4	7.15	7.18	+0.03

saturated at a tension fairly near it. Fig. 2 was constructed as follows. About fifty observations on carbon dioxide content and tension of miscellaneous pathological and normal human cases

were secured.[1] There is a general tendency for carbon dioxide tension and carbon dioxide content to vary together, but the scattering is so great that it is impossible to express this tendency accurately. The average *AB* line was, therefore, drawn. The twenty abnormally low values (below 36 mm. of carbon dioxide tension) were averaged and the corresponding carbon dioxide

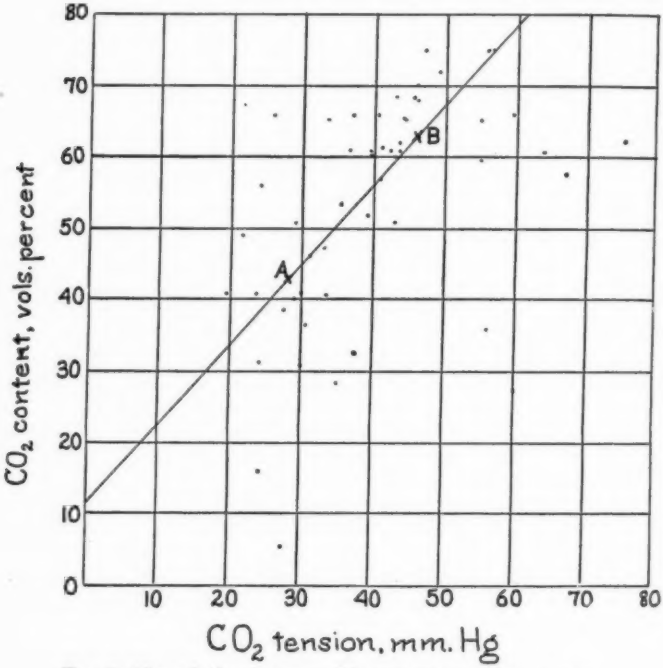

FIG. 2. The relation between CO_2 content and CO_2 tension.

content values were averaged. These average values were used to locate point *A*. Similarly, the thirty values above 36 mm.

[1] Only about ten of these determinations were done according to the technique described in this paper. The remaining data were obtained from colorimetric determinations and are, therefore, not strictly accurate. They probably serve well enough to determine the average relation between carbon dioxide content and tension.

tension were averaged and used to locate point *B*. The line *AB* naturally affords only a rough means of estimating pH. There was much scattering among the low tension cases (in many of them the carbon dioxide content was relatively high), but even then, the error in using the chart rarely exceeds 12 mm. In the tension range from 36 to 60 mm., the error in using the chart rarely exceeds 8 mm. The carbon dioxide content corresponding to the two uppermost tensions (64 and 75 mm.) was relatively low, and the error is large, but these are extreme cases.

This chart, it may be added, can be used to approximate the carbon dioxide tensions of the original serum only for human sera. pCO_2 and pH relative to carbon dioxide content were found to be lower for cat sera (16) than for human sera; hence it would probably be necessary to construct a chart for each species.

Determinants of the Separated Serum or Plasma Slope.

Although the variations in (Δ $CO_{2_{60-37}}$) are not of enormous magnitude, still there is enough scattering to permit the hope that the conditions causing these variations might be indicated.

There are several experiments designed to show the influence of bicarbonate concentration on (Δ $CO_{2_{60-30}}$). Table V shows the nature of the specimen used and other determinations or observations made on a given sample. In Cases 1, 2, 3, and 8, true plasma was separated at 30 mm. of carbon dioxide tension and saturated at 60 mm. (specimen A), true plasma from the same blood specimen was separated at 60 mm. and saturated at 30 mm. (specimen B).

In each case (Δ $CO_{2_{60-30}}$) was greater for specimen B than for specimen A. The 60 mm. true plasma has a higher bicarbonate concentration and a lower pH than the 30 mm. true plasma, factors which probably account for the increased (Δ $CO_{2_{60-30}}$) in specimen B. There is one slight variable here that should possibly be considered. 60 mm. true plasma is more concentrated than 30 mm. true plasma because the cell volume at 60 mm. is increased by water derived from the serum under the influence of the greater acidity of the blood at the higher tension of carbon dioxide. The carbon dioxide content of 60 mm. true serum is, however, so little increased by this factor, that it could hardly account for the greater value for (Δ $CO_{2_{60-30}}$) in specimen B. In any one plasma, then, the steepness of the slope seems to vary with the bicarbonate

TABLE V.

Separated Serum or Plasma Slopes, ($\Delta CO_{2_{60-30}}$), and Its Determinants.

Case No.	Separated serum or plasma carbon dioxide content at: 30 mm. CO_2 tension.	Separated serum or plasma carbon dioxide content at: 60 mm. CO_2 tension.	($\Delta CO_{2_{60-30}}$)	Plasma or serum proteins.	Remarks.
	vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	
1	50.5	55.7	5.2	6.94	True plasma separated at 30 mm., resaturated at 60 mm.
	59.65	65.0	5.35		True plasma separated at 60 mm., resaturated at 30 mm.
2	43.7	48.6	4.9	8.67	True plasma separated at 30 mm., resaturated at 60 mm.
	53.8	59.95	6.15		True plasma separated at 60 mm., resaturated at 30 mm.
3	46.75	52.45	5.7	8.57	True plasma separated at 30 mm., resaturated at 60 mm.
	56.75	62.7	5.95		True plasma separated at 60 mm., resaturated at 30 mm.
4	46.85	51.2	4.35	7.81	True plasma separated at 30 mm., resaturated at 60 mm.
	25.6	32.05	6.45		0.01 cc. concentrated lactic acid added to 12 cc. of 30 mm. true plasma, acidified plasma then separated and saturated at 30 and 60 mm.
5	54.25	58.6	4.35	7.01	True plasma separated at 30 mm., resaturated at 60 mm.
	65.8	70.3	4.5		5 mg. of Na_2CO_3 added to 15 cc. of 30 mm. true plasma, alkalinized plasma then separated and saturated at 30 and 60 mm.
6	62.7	69.55	6.85		True plasma separated at 30 mm., resaturated at 60 mm.
	50.45	56.35	5.9		0.01 cc. of concentrated lactic acid added to 12 cc. of 30 mm. true plasma, acidified plasma then separated and saturated at 30 and 60 mm.
7	45.75	51.55	5.8	7.53	True plasma separated at 30 mm., resaturated at 60 mm.
	28.8	33.7	4.9		0.04 cc. of concentrated lactic acid added to 36 cc. of whole blood, then true plasma of acidified blood saturated at 30 mm., then separated and saturated at 60 mm.

TABLE V—*Concluded*

Case No.	Separated serum or plasma carbon dioxide content at: 30 mm. CO_2 tension.	Separated serum or plasma carbon dioxide content at: 60 mm. CO_2 tension.	(ΔCO_2 60–30)	Plasma or serum proteins.	Remarks.
	vols. per cent	*vols. per cent*	*vols. per cent*	*vols. per cent*	
8	58.6	61.5	2.9	7.54	True plasma separated at 30 mm., re-saturated at 60 mm.
	59.0	65.4	6.4		True plasma separated at 60 mm., re-saturated at 30 mm.
9	49.5	54.4	4.9	7.23	True plasma separated at 30 mm., re-saturated at 60 mm.
10	69.55	75.0	5.45	6.84	True plasma separated at 60 mm., re-saturated at 30 mm.
11	42.8	49.5	6.7	7.09	True plasma separated at 30 mm., re-saturated at 60 mm.
12	27.7	33.4	5.7		True plasma separated at 60 mm., re-saturated at 30 mm.
13	42.1	48.3	6.2	7.08	Separated serum saturated at 20 and 60 mm.
13A	46.2	51.7	5.5	6.89	" " " 20 " 60 " (13A 7 days after 13).
14	55.5	60.7	5.2	6.74	Separated serum saturated at 30 and 60 mm. (normal).
	55.6	60.0	4.4	6.64	Separated serum saturated at 30 and 60 mm. (after O_2-want).
14A	59.4	65.7	6.3	6.36	Separated serum saturated at 30 and 60 mm. (normal).
15	59.45	65.95	6.5	7.36	Separated serum saturated at 30 and 60 mm. (normal).
	58.3	65.5	7.2	7.56	Separated serum saturated at 30 and 60 mm. (after O_2-want).
16	52.25	58.45	6.2	6 68	Separated serum saturated at 30 and 60 mm. (normal).
	65.8	71.15	5.35	6.04	Separated serum saturated at 30 and 60 mm. (after bicarbonate injection).
16A	56.9	65.9	9.0	7.43	Separated serum saturated at 30 and 60 mm. (normal).
	63.8	71.75	7.95	6.66	Separated serum saturated at 30 and 60 mm. (after bicarbonate injection).
17	59.8	62.8	3.0	5.63	Separated plasma saturated at 30 and 60 mm. (pneumonia).
18	57.1	63.15	6.05	6.76	Separated serum saturated at 30 and 60 mm.

concentration. No general correlation between bicarbonate content and the magnitude of the value for ($\Delta CO_{2_{60-30}}$) was observed in comparing plasmas from different subjects.

Studies were made of the effect of changing pH and bicarbonate concentrations by adding lactic acid or sodium carbonate to true plasma or whole blood. The results of the addition of lactic acid may be observed in Cases 4, 6, and 7. In Cases 4 and 6, 0.01 cc. of concentrated lactic acid was added to 12 cc. of 30 mm. true plasma. The slopes of the unmodified and the acidified separated plasmas were determined. In Case 7, 36 cc. of whole blood were centrifuged and 0.04 cc. of concentrated lactic acid was added to the supernatant plasma. The cells and plasma were mixed again. Another sample of the same blood was treated in like manner, except that 0.04 cc. of distilled water was added instead of the acid in order to make the control of the same dilution as the acidified sample. 30 mm. true plasmas of both samples were obtained, then the plasma of each was separated and saturated at 60 mm. The effects of the addition of lactic acid are not consistent. In Case 7 its addition (to true serum) causes a decrease in ($\Delta CO_{2_{60-30}}$); in Case 4 a marked increase. In Case 7 its addition to whole blood causes an appreciable fall in ($\Delta CO_{2_{60-30}}$). The effects of the addition of sodium carbonate may be observed in Case 5. 5 mg. of sodium carbonate were added to 15 cc. of 30 mm. true plasma; the effect on ($\Delta CO_{2_{60-30}}$) was hardly appreciable.

The addition of lactic acid or sodium carbonate to whole blood or true plasma produces changes other than simple alteration of bicarbonate concentration. The addition of a non-volatile, non-buffering acid such as lactic to true serum is not comparable to lowering the pH by increasing carbon dioxide. The former causes a decreased pH, but carbon dioxide is also decreased. The addition of lactic acid to whole blood undoubtedly entails a readjustment of bicarbonate and chloride distribution between cells and plasma. The addition of sodium carbonate to true serum increases bicarbonate, increases pH, and introduces still another variable because it increases base concentration.

The response to *in vivo* alterations of acid-base equilibrium was also studied, with no more consistent results. The separated serum slope of one normal individual (Case 14) after oxygen-want

is decreased, that of another (Case 15) is increased. There are two experiments on one normal (Cases 16 and 16A) where the slope was determined before and after bicarbonate injection. There was a drop of about 1 volume per cent of carbon dioxide in both experiments. Of course the response to bicarbonate is hardly comparable to the effects of the *in vitro* addition of the salt to true serum. In the one case, extravascular changes alter the whole ionic equilibrium of the cells and serum. In the case of the *in vitro* addition of the salt to true serum, all the base added remains in the serum, and there are no cross membrane redistributions to affect the results.

In a limited number of cases, data on cell volume, oxygen capacity, serum sodium chloride, true plasma slope, and protein content were secured. Since the variations in (Δ $CO_{2_{60-30}}$) seem to have no relation to variations in any one of these components, the results are not given. The number of variables is so large in proportion to the number of experiments that any relation to one component might well be obscured by variations of the others. It is, for example, hardly conceivable that protein concentration should be without influence on the grade of the slope; but protein content, in the sera of different individuals, does not seem to be correlated with the value for (Δ $CO_{2_{60-30}}$). If, however, the paired Cases 13 and 13A, 14, 15, 16 and 16A are considered, it may be observed that for any one case (Δ $CO_{2_{60-30}}$) varies in the same direction as, but not in quantitative proportion to, the protein content. In other words, where the other constituents of a serum may be assumed to be approximately the same, as in the case of the serum of any one individual, protein content does qualitatively influence (Δ $CO_{2_{60-30}}$); the failure to secure quantitative correlation is probably due to changes in constituents other than protein.

SUMMARY.

1. A gasometric technique for the determination of pH in serum or plasma is described. This method is capable of wider application; and is more accurate than the colorimetric methods of Cullen and of Hastings and Sendroy and is less complicated than the combination colorimetric and gasometric method described by Austin, Stadie, and Robinson.

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2. The method consists in determining the carbon dioxide content of serum from blood drawn and centrifuged without air contact. Other samples of the separated serum are saturated at two different carbon dioxide tensions (one on either side of the content point). The saturated sera are also analyzed for carbon dioxide. By means of the logarithmic pH chart described elsewhere the carbon dioxide absorption curve is constructed and on this the carbon dioxide content of the unknown blood is located. pH can then be read off directly or can be mathematically calculated by means of formulæ.

3. A less accurate method, which, however, possesses the advantage of requiring less serum is described. In this method only one point is used for the estimation of the CO_2 absorption curve.

4. The determinants of the separated serum slope are discussed. Bicarbonate and protein were the only constituents investigated which had a demonstrable effect on the value for (Δ $CO_{2_{(30-20)}}$), which seemed to vary in the same direction as these constituents.

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ACTIVITY IN PROTEIN SOLUTIONS.

I. INERT GASES. THE QUESTION OF HYDRATION.*

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INTRODUCTION.

This paper is part of an attempt to determine activities in protein solutions, and their possible relation to the question of hydration of proteins. Here are detailed experiments on one of the simplest cases—the solubility of an inert gas in a pure protein solution—and the results are discussed in the light of other work on gas solubility in solutions.

I. Previous Attempts to Determine Hydration from Gas Solubility.

1. Philip.

The solubility of hydrogen in solutions of electrolytes and non-electrolytes was considered by Philip. From the solubility of the gas per liter of solution and the amount of water per liter of solution he calculated the solubility of the gas per liter of water in the solution. The difference between this figure and the solubility in pure water divided by the solubility in pure water was considered to give the fraction of water "bound." From this was calculated the water bound per molecule of solute. The following equations were formulated from the examples given in his paper:

Let a = solubility of gas in cc. per liter of solution.
" a_w = " " " " " " " " " pure water.
" a_{ws} = " " " " " " " " " water in solution.

* Construction of apparatus aided by a grant from the Proctor Fund, Harvard Medical School.

Let w = per cent of water in solution (per 100 gm. of solution).
" w_{cc} = " " " " " " " 100 cc. " "
" s = " " " solute " " " 100 gm. " "
" s_{cc} = " " " " " " " 100 cc. " "
" D = density of solution.
" M_w = molecular weight of water.
" M_s = " " " solute.
" w_b = fraction of water bound.

Then $s \times D = s_{cc}$

$(100 \times D) - s_{cc} = w_{cc}$

$\frac{100 \times a}{w_{cc}} = a_{ws}$

$\frac{a_w - a_{ws}}{a_w} = w_b$

$\frac{w_b \times w \times M_s}{s \times M_w}$ = mols bound per mol of solute.

Philip found hydration in sucrose solutions as follows:

Per cent of sucrose by weight.	Mols of water of hydration per mol of sucrose.
16.67	6.5
30.08	6.0
47.65	5.4

Small errors are apparent in Philip's figures. His data are stated to be from Knopf, but actually are from Gordon, and apply to solubility at 15°C. instead of 20°C. The correction of this error does not materially change the results.

These figures for the hydration of sucrose seem to check fairly well with other methods of calculation. Philip also gives data for chloral hydrate, KCl, and several inorganic substances, with calculated hydration in each instance.

2. MacArthur.

MacArthur, using O_2, carried out similar experiments and calculations. His experiments with sucrose were nullified by reaction of the oxygen with the sucrose.

3. Lewis and Associates.

Lately Lewis has used a similar calculation to determine the relation of activity to hydration. He worked with 0.1 N and 0.5 N KCl and H^+ from 0.1 N HCl by means of cells with trans-

port. The H^+ measurements checked with direct E.M.F. measurements with the hydrogen electrode.

These experiments gave some very interesting results. KCl showed an activity equal to that calculated from the amount of water in the solution, without assuming any hydration of the sucrose. The H^+ showed greatly increased activity, in 60 per cent sucrose amounting to over twice the activity of KCl. Later experiments with HCl by the potentiometer method in the cell

| N calomel electrode | saturated KCl | 0.1N HCl : sucrose | H_2 |

gave an increase in activity of H^+, only about two-thirds of which could be accounted for by the decrease in the water due to the presence of the sucrose, even assuming 4 mols hydration. After a still further allowance for increased activity calculated from the heat of dilution, the activity still remained greater than calculated, as is evident from the following figures:

Concentration of sucrose. Mols per liter.	aH relative.	aH calculated.
0	1.00	1.00
0.877	1.74	1.44
1.755	3.00	2.34
2.047	3.60	2.89

According to Lewis, only by assuming greater ionization of the HCl, for which there is no evidence, could the calculated figures be made to check with the observed.

Experiments on the activity of H^+ from acetic acid in sucrose solutions led to the conclusion that the observed increase in activity could be accounted for by assuming an hydration of $4H_2O$ for the sucrose, of $3H_2O$ for the acetic acid, and no increase in ionization of the acetic acid in the presence of the sucrose. The latter assumption, of course, is scarcely harmonious with the assumption of an increase in ionization of the HCl in the presence of the sucrose.

Ba^{++} in $BaCl_2$ was found to have an activity accounted for by allowing for a decrease in water due to the sucrose, but assuming no hydration of the sucrose whatever.

Studies on the activity of H^+ from HCl in causing the conversion of hydroxyvaleric acid into valerolactone resulted in conclusions

that the hydroxyvaleric acid molecule is not soluble in the apparent water of hydration of the sucrose while it is in the water of hydration of KCl or LiCl. H^+, however, was not soluble in the water of hydration of any of these.

Thus the study of activities has led to puzzling results, when the authors tried to interpret them from the foregoing point of view. Many other papers of similar nature cannot be entered into here.

4. Source of Error in Foregoing Calculations of Hydration.

Even a short consideration of such methods of calculation seems to show a lack of appreciation of a factor which is obviously of theoretical importance, and can be proved in other simpler cases to be of practical importance.

The following hypothetical case will show the point involved: Suppose we determine the activity of a perfect gas "g" in a solution of two miscible liquids showing no heat effect or volume change on mixing, and designated as l_1 and l_2. Let the solubility of the gas in l_1 be twice its solubility in l_2. If in any given cases one calculates the pressures necessary to bring the solubilities back to some constant figure as standard, the pressures will be in inverse relation to the solubilities. The relative activity compared to that in the standard will be in direct proportion to the pressure, so that the relative activities will be in inverse relation to the relative solubilities. In this case, for convenience, the activity in the component giving the greatest solubility will be chosen as the standard, or l_1. Take for example 100 cc. of a 50 per cent mixture of the components.

If solubility in $l_2 = a$,
then " " $l_1 = 2a$.

In 50 cc. of l_2 the solubility is $\frac{a}{2}$.
" 50 " " l_1 " " " a.
" 100 " " mixture " " " $\frac{3a}{2}$.
" 100 " " " " " relative to that in $l_1 = \frac{3a}{2} \div 2a = \frac{3}{4}$.
" 100 " " " " activity " " " " $l_1 = \frac{4}{3}$.

By the methods in the literature the calculated activity relative to l_1 as solvent would be as follows:

In 50 cc. of l_1 the solubility is a.

" 100 " " a 50 per cent mixture the relative solubility is $a \div 2a = \frac{1}{2}$.

" 100 " " "50 " " " " " activity = 2.

Thus, allowing for solubility in both constituents results in a calculated activity $\frac{4}{3} \div 2 = \frac{2}{3}$ of that calculated by the other method.

This proves that theoretically great allowances may have to be made in many cases for solubility or activity in the solute as well as in the solvent.

If the activity of $\frac{4}{3}$ were found to be the observed activity, then the calculation by the methods in the literature, giving an activity of 2, would, by the reasoning in the literature, necessitate a large negative hydration. Examples of this in actual cases will be given later. Solubility in the solute will always act to diminish any "hydration" which may otherwise be apparent, and cannot be neglected as a factor unless proved in some way to be negligible. It is probably unimportant in the case of the solubility of H_2 in sucrose or inorganic salts, but there are other difficulties in the way of Philip's reasoning from gas solubility to hydration, which will be explained later.

Whether ions may not have an important solubility in sucrose is not known, and it seems possible that part of the discrepancies noted by Lewis may be explained by different solubilities of the third component in the solute, rather than in a hypothetical water of hydration.

Since the observed activity of Lewis for the third constituent giving the greatest activity (*i.e.*, H^+) is too great for the calculated activity, it would be necessary to assume a greater hydration than 4 for sucrose, even with zero solubility for H^+ in sucrose, or else to conclude that further factors enter in. Therefore the following analysis was made of the relation of activity to hydration.

II. Activity and Hydration.

1. What Is Meant by Hydration.

The term "hydration" or in a broader sense "solvation" seems to be used to cover cases similar to the following: (*a*) Definite compound formation, the compounds being capable of isolation; (*b*) definite compound formation, the compounds being incapable of isolation; (*c*) indefinite compound formation—a sort of attraction and holding of water by the solute; (*d*) "adsorption" of water by the solute.

The variety under (*c*) offers difficulties. It would appear to be identical with an increased internal pressure, which means an increased attractive force between the solute and solvent. Suppose in another case the internal pressure decreases, as it sometimes does, then we must call it "negative hydration" or else discard this definition of hydration. The latter course will be adopted here.

As far as relations in solution go, the question of the possibility of isolating the compound does not enter in, so that (*a*) and (*b*) may be considered together. The variety (*d*) is merely compound formation on surfaces of the solute, and would differ from (*a*) only in quantitative relation to concentration. Since this will be largely neglected in this paper, it may be put under (*a*).

This reduces "hydration" for present purposes to: definite compound formation between solute and solvent.

2. Factors in Activity of a Third Component in Solutions of Two Components.

a. Composition of the solution.

b. Solubility in the amount of each pure component present.

c. Change in its solubility in the solution from that calculated from the composition on account of: (1) Interaction of the components, forming compounds (solvates): (*a*) Thus diminishing the amounts of the components uncombined, and decreasing the solubility over that calculated for a mixture; (*b*) necessitating consideration of solubility in the compound formed (on account of changes of polarity, etc.); (*c*) changing the internal pressure of the solution over that due to simple mixing on account of different attractive power of the compound; (*d*) necessitating consideration

of solubility in the water of hydration. (2) Interaction of the components, changing the internal pressure over that calculated for the mixture, without forming definite compounds. (3) Association or dissociation or depolymerization of one or both of the components: (*a*) Changing the number of mols (or ions); (*b*) changing the solubility of the third component in the altered component per mol, on account of altered polarity, internal pressure, electrical attraction, etc.; (*c*) changing the internal pressure of the solution. (4) Interaction of the components, changing their polarity, without forming definite compounds. (5) Adsorption of the third component by one of the components.

3. Relation of Activity Changes to Hydration.

It is evident from this outline of obviously possible interrelations between activity and hydration, that most of them are never considered at all. It is also evident that it will be a difficult task in any given case to determine the presence of solvation, and still more so to get a definite figure for its degree, from any determinations of activity alone, until the problem has been simplified. In Philip's examples, for instance, the internal pressure of sucrose is not known, nor the internal pressure of the solutions. If the internal pressure of sucrose is high, its mere presence would probably increase the internal pressure of the solution and lower the solubility of H_2. There is a further possibility that the internal pressure of the solution may be increased beyond that due to simple mixing, with a further reduction of solubility. These corrections may entirely account for the lowered gas solubility, without any hypothesis of hydration.

On account of the complexity of the problem, all that will be attempted here will be an elimination of minor factors; a bringing to prominence of what appears to be the major factors; and, in general, a qualitative consideration.

4. Simplification of the Relation of Activity to Hydration. Special Cases.

The problem was attacked in the first place by choosing such conditions that as many factors as possible would be eliminated. Suppose "*a*" and "*b*" to be known, as it is easily possible to arrange

for. If two-phase systems are left aside, "*c* (5)," or adsorption, is out of consideration. If the third component is inert, "*c* (4)," or the influence of changes of polarity, is reduced to a minimum. Further, if the third component has an internal pressure far from that of the solution components, the relation of internal pressures is simplified. These considerations led to the choice of an inert gas as the third component. Other reasons for this choice are that it is soluble in such small amounts that, together with its inertness, the effect on the solution will be practically infinitesimal.

There are left factors (1), (2), and (3). Suppose we can estimate roughly the change in internal pressure by means of surface tension measurements, for instance, then factors (1) (*c*), (3) (*c*), and (2) become known as a summated effect, although not separately. Suppose further that the solutions are chosen to eliminate dissociation.

In the case of water solutions Richards has given us a means of dealing with the polymerization changes in the water. These he estimates as about 2.8 per cent of the gm. of water present for the addition of each mol of solute. The following calculation gives the change in solubility due to the change in the number of mols of water.

Per Cent Change in No. of Mols of Water Due to Addition of 1 Mol of Solute at 20°C.

$(H_2O)_2$ per 100 gm. of water.		H_2O per 100 gm. of water.		Total mols.	Change in mols.	Per cent change.
Before addition of solute.						
gm.	*mols*	*gm.*	*mols*			
28	0.777	72	4.0	4.77		
After addition of solute.						
25.2	0.70	74.8	4.15	4.85	0.08	1.7

Therefore if the change in activity in a 1.0 M water solution is much greater than that calculated for a 1.7 per cent change in the number of mols of water present, the factor due to depolymerization of the water can be assigned a place as a modifying factor only.

The factors now left are: (1) (*a*) Decrease in solubility due to formation of a compound reducing the number of mols; (1) (*b*) solubility in the compound; (1) (*d*) solubility in the water of hydration; (3) depolymerization of any component but water (*a*) changing the number of mols, (*b*) changing the solubility per mol.

This may be simplified further by assuming that the solubility in the compound is not much different than in the components, and that the solubility in the water of hydration is taken care of by this factor of solubility in the compound formed. These seem conservative assumptions for purposes of qualitative consideration. Let us make a similar assumption about solubility in the depolymerized component.

The net result of the analysis for the special case is:

Known quantitatively:
- Composition of the solution.
- Solubility in each pure component.
- Depolymerization of water.

Known roughly:
- Change in internal pressure.

Unknown:
- Formation of a solvate, decreasing the solubility as far as the reduction in the number of mols is concerned.
- Depolymerization of solute, increasing the solubility as far as the number of mols is concerned.

Therefore it appears probable that if we have a solution of an inert third component in a mixture of non-dissociated liquids, and find a decrease in solubility over that calculated for the mixture so large that it cannot be accounted for by the change in internal pressure (especially if the change in internal pressure is in the wrong direction) we can say it is not due to depolymerization, for this would increase the solubility, but it must be due to the formation of a compound.

If we have a decrease in solubility consistent with an increase in internal pressure, there may or may not be solvation.

In either case depolymerization may also be present, tending to diminish the apparent amount of compound formed.

An increase in solubility with no decrease or an increase in internal pressure would indicate depolymerization.

Further light might be thrown on the situation by considering the volume change on mixing the components. Since solvation means that the affinities between the components are greater than between the molecules of either component, the formation of a solvate would result in a contraction in volume which might not be accompanied by an increase in internal pressure of the solution as a whole. It may be well to state here that by "internal pressure" is meant intermolecular attractive force. It is evident that it is changes in the force holding the molecules together in the solution which will be important in causing deviations in solubility. On account of high chemical affinity between the solution components, a compound may be formed with contraction in volume and decrease of compressibility, but, after it is formed, the affinities of the components making it up will be relatively satisfied, and less free to attract other molecules. Hence one would expect a tendency to a decrease, probably slight, of intermolecular attraction in the case of solvate formation.

A contraction of volume would also result from an increase in internal pressure over that due to simple mixing, with decreased solubility.

Depolymerization with decrease in volume, as in the case of water, would cause increased solubility, unless the depolymerized water has a higher internal pressure. This latter appears probable, in which case here too there would be contraction in volume and a decrease in solubility, or a neutralization of the increase due to the greater number of mols.

In general, then, contraction in volume would mean decreased solubility of a third component of low internal pressure.

An expansion would mean a decrease in internal pressure, or depolymerization with expansion, as in the case of alcohol. Here it seems probable that the depolymerized molecules would have less internal pressure, which would give an increase in solubility.

Richards gives besides the forces of attraction, and depolymerization, the compressibilities of the substances as a factor in the amount of volume change. In relation to the solubility of an inert gas, this factor has less importance than at first appears. Suppose, for instance, there is a certain volume change A in the case of two substances both of high internal pressure and therefore relatively incompressible, and the same change A in the case of

two others both of low internal pressure. In the first case there would undoubtedly be a greater force of affinity involved, and a greater increase in internal pressure (in absolute amount), but this increase would be on a higher internal pressure to begin with, so that the result in both cases would tend to be a per cent increase in internal pressure of the same magnitude. It is the per cent increase that is important in affecting the activity.

Conclusion.—The following are the main possibilities with the corresponding changes in the factors from those calculated for mixtures: (1) Increased internal pressure, contraction of volume, decreased solubility. (2) Solvation, contraction of volume, decreased solubility, internal pressure about same. (3) Solvation, contraction of volume, decreased solubility, internal pressure decreased but not enough to counteract decreased solubility. (4) Solvation, contraction of volume, decreased solubility, internal pressure increased. (5) Depolymerization, contraction of volume, decreased solubility, internal pressure increased. (6) Depolymerization, contraction of volume, increased solubility, internal pressure about same. (7) Depolymerization, contraction of volume, increased solubility, internal pressure decreased. (8) Solvation, contraction of volume, increased solubility, internal pressure much decreased.

Evidently varieties (2) and (3) can be distinguished, but (4) cannot be told from (1) or (5), but as stated above, probably (6) and (7) do not occur.

The tabulation for expansion of volume is omitted.

III. Correlation between Activity and Volume Changes in Solutions.

1. In order to test the probable relation between volume change and activity, the activities of inert gases in solutions of miscible organic liquids were calculated. The ratio of the activity observed to the activity calculated from the solubility in the pure components was compared to the ratio of the volume of the solution calculated from the amounts and densities of the pure components to the volume observed; *i.e.*, the correlation between $\frac{\text{activity observed}}{\text{activity calculated}}$ and $\frac{\text{volume calculated}}{\text{volume observed}}$. The term "inert

gas" is here used to indicate a gas of low internal pressure and polarity which is inert as far as chemical activity in the solutions in question is concerned. Sulfuric acid has been included in the solutions, the calculations being made on the basis of complete dissociation.

2. Raoult's Law.—It is evident from I (4) that Raoult's law is of little use in considering solutions from this point of view. When we calculate the solubility in the amount of the pure component present we do actually include all that we should have if we used Raoult's law, and in addition we allow for the solubility in the pure components. In other words, deviations in activity from the mixture law are studied rather than deviations from Raoult's law.

3. Methods of Calculation.—It is considered that in comparison with experimental errors in determining gas solubility, errors due to omission of corrections of deviations of the gases from the perfect gas law will be negligible for the small variations in pressures involved in calculating solubilities to a standard. It will make no difference whether the Bunsen coefficient is used or the Ostwald, provided the same one is used in calculating both parts of the ratio.

The volume changes are calculated from the densities of the pure components, and the composition and density of the solution.

In considering the data and charts it must be remembered that the determinations of gas solubility in many cases do not have a high degree of accuracy. The density figures are of greater accuracy, but much smaller variations need to be considered. The volume ratio has been plotted on a different scale from the activity ratio, for both theoretical and practical reasons. In the case of each kind of solution the volume change has been calculated for one temperature only. In some cases the solubilities by different workers vary over a small temperature range. It is possible that calculations of volume change for each temperature would show a closer correlation.

The data are given in two parts: (*a*) where they are apparently satisfactory, (*b*) where they are not entirely satisfactory. The latter cases are those where densities at the same temperature as the gas solubilities were unobtainable. This introduces an error into the gas activity calculations. Where the temperature

difference is not over 5°C. it is believed that it only slightly modifies the results.

4. Data on Correlation between Activity and Volume Changes in Solutions. (*See Tables I to XVI and Charts I to XV.*)—In each case weight per cent means gm. per 100 gm. of solution. Except where otherwise indicated on the chart, the volume ratio is given as a dotted line with *X* for the points. λ in the tables means the Ostwald absorption coefficient, and B the Bunsen coefficient referred to the volume of the solution. (Sometimes the Bunsen coefficient is used to refer to the weight of solution.) $\frac{\text{Activity observed}}{\text{Activity calculated}}$ and $\frac{\text{volume calculated}}{\text{volume observed}}$ refer to the activity and volume ratios respectively.

5. Discussion of Data. Glycerol-Water Solutions (*Tables I to V, Charts I to IV*).—The activity ratios of H_2 and N_2 calculated from the solubility data of Muller, Drucker, and von Hammel, are shown in Charts I and II. (Each curve is plotted with the initial of the experimenter for the points.) In spite of variations in the degree of inflexion of the curves, depending on the experimenter, there is general parallelism of the curves. The activity ratios of O_2 and CO_2, given in Tables IV and V, but not charted, show similar curves. The activity ratio curves are as a whole parallel to the volume ratio curve, with the exception of the maximum point, which is at 57.5 per cent for the volume ratio and about 66 to 70 per cent for the activity ratios.

In Chart I a curve is given showing the volume curve corrected for the effect due to the depolymerization of water according to Richard's conclusions. That is, the curve shows the volume change omitting that due to depolymerization. The maximum shifts from 57.5 to 60 per cent, thus bringing it nearer to the activity ratio curve. There is probably a considerable increase in internal pressure in the solutions, which would increase the depolymerization, thus bringing the maximum still nearer the activity ratio maximum.

Correction of the activity figures for the increase in solubility due to the increase in number of mols from depolymerization would not greatly affect the ratio. In a 50 per cent solution, for instance, solubilities in the amounts of glycerol and water present are about equal. An 0.85 per cent change in solubility in the water (this is

the amount due to depolymerization from the addition of $\frac{1}{2}$ mol of solute) would make a change of only about 0.43 per cent in the activity and its ratio. The lowest activity ratio of the various observers for this concentration was 25.2 per cent above 1.00. Correction of the gas activity curve for this effect would tend to throw the lower part of the curve further to the right and lower the maximum, thus bringing it closer to the volume ratio.

The variation in the degree of inflexion of the curves with different experimenters may depend on errors which are constant for each experimenter. If so, it would be worth while to compare the results with different gases for each experimenter separately. Such comparison shows that the degree of inflexion is greater the less the solubility of the gas, with the exception of O_2, which is not too far out of place to be the result of experimental error.

Chart III shows the activity ratio calculated on the basis of the total water present. The great divergence begins in concentrations over 50 per cent.

Chart IV demonstrates that calculation of "hydration" by the method of Philip, etc., gives a negative hydration in the higher concentrations even in the case of such a mixture as this with greatly decreased gas solubility. In solutions where there is less of a decrease in solubility the negative hydration would be more marked, and it would be present in all concentrations where the solubility in the added component is greater than in the "solvent" and the internal pressure is the same or decreased.

Charts V to VII and Tables VI to VIII give the activity and volume ratios of CO in three mixtures of organic liquids. There is general correlation in the ratios. It is of interest that in Charts V and VI there is expansion of volume and increase of solubility. Increased solubility in solution does not necessarily mean adsorption.

Chart VIII and Tables IX and X show the activity ratios of CO and H_2 in alcohol-water mixtures. There are technical difficulties in these gas solubility determinations, and the curves are evidently crude. The direction of variation is the same however. The maximum of the activity ratio is lower than of the volume ratio.

Chart IX and Table XI show an extremely close correlation between the activity ratio calculated from the solubility of N_2 in

H_2SO_4 by Bohr and the volume ratio. Chart X and Tables XI and XII give ratios for N_2 and H_2 from figures by Christoff. The correlation is not as close and the maximum is slightly lower for the activity ratio, but there is general parallelism.

Chart XI and Table XIII, of the ratios of CO in ethyl alcohol-benzene mixtures, are of considerable interest. Both curves shift from one side to the other of 1.00, on account of an expansion of volume in the lower concentrations and a contraction in the higher ones.

Charts XII to XV and Tables XIV to XVII include the solutions where the densities used for the gas activity calculations were for a different temperature. The first two solutions show correlation of the ratios, but the last two show definite lack of correlation. Whether this is due to the fact that the densities are 9°C. different cannot be stated.

Conclusion.—The activity ratios of gases of low internal pressure and polarity in a variety of mixtures of organic liquids, and in one inorganic liquid mixture where complete ionization is assumed, show a general tendency to vary with the corresponding volume ratios. The degree of inflexion of the curves may not be the same, but they are in general parallel. In certain cases where the maxima are not coincident, a closer correlation is obtained by estimating the effect on volume change due to depolymerization of water, and subtracting this from the total volume change.

IV. Internal Pressure Changes in Relation to Data of Part III.

The surface tension was chosen as an indication of the trend of internal pressure changes. Various authors have attempted to estimate the actual amount of internal pressure from the surface tension. There are several objections to these calculations. The surface tension decreases as the substance of lower surface tension concentrates in the surface, so that only values made instantaneously on a perfectly fresh surface would be acceptable. The surface tension does not accurately represent intermolecular attractions in the interior of the liquid, in many cases, because polar groups tend to gather in the surface layer, giving an attractive force not representative of the molecule as a whole. Further, some of the equations calculate the absolute attractive force,

allowing for the distance between molecules, while what is desired in questions of solubility is not the absolute attractive force between two molecules, but the attractive force that exists between the molecules at the distance at which they are placed in the liquid.

Surface tension as ordinarily calculated is per unit length and varies with the length in direct ratio. To get the force in terms of attraction between a pair of molecules in the surface, the surface tension should be divided by the number of molecules per unit length or the cube root of the number of molecules per unit volume. This figure has been used in the following calculations and termed the surface tension per mol.

Chart XVI shows the solubility per mol of N_2 in H_2SO_4 (from Bohr's figures) plotted against the surface tension and the surface tension per mol. There is a considerably closer relation between the solubility per mol and the surface tension per mol. Deviations from the mixture law are shown in such a plot by deviations from a straight line between the pure components. Here increased internal pressure goes with decreased solubility. The maximum of the internal pressure curve, however, is at about 53 per cent, while the maximum of the decrease in solubility is at about 77 per cent. According to the previous analysis this decrease in solubility from the 53 per cent concentration to the 77 per cent while the internal pressure is decreasing shows solvate formation. The contraction in volume continues over this same range. It does not seem likely that at this concentration the errors previously mentioned as possibilities in surface tension deductions would be at all large. Correction for depolymerization would affect the solubility per mol and the surface tension per mol curves equally.

A similar relation is apparent in the first part of the curve between 0 and 6 per cent. In these solutions the tendency would be for a decrease in concentration in the surface, and this would be more apparent in the lower concentrations. The result would be to decrease the effect of the solute on surface tension, thus making a smaller increase in surface tension than one would expect. Hence the lack of increase in surface tension in the more dilute solutions. Contraction in volume is as marked here as decrease in solubility. Therefore solvate formation is probable.

The conclusion from the whole curve is that there is solvate

formation throughout, but that in low concentrations not enough solute has been added to affect the internal pressure of the solution as a whole. In concentrations from about 6 to 53 per cent there is increase both in internal pressure and solvate formation on account of the large amount of solute present, only part of which is combined, while from 53 to 77 per cent solvate formation is extensive enough to use up a large part of the added solute and prevent a further increase in internal pressure.

Chart XVII shows plots for chloroform-acetone solutions. The surface tension per mol curve and the solubility per mol curve run in the same direction except for the terminal portions which may be due to experimental errors, since the surface tension figures appear to be of low accuracy.

Chart XVIII gives curves for alcohol-water solutions. Up to about 30 per cent the solubility per mol decreases while the surface tension per mol decreases, and decreases more than would result from simple mixing. No errors of the kind discussed would affect the direction of the internal pressure change deduced from the surface tension where the change is so marked as this. From Chart VIII it is seen that contraction in volume is marked over this range. The conclusion is that solvate formation is marked. Apparently cases like this show rather definitely that internal pressure does not, by any means, necessarily increase when there is contraction in volume.

From 30 to 50 per cent there is further contraction in volume, increase in solubility, and decrease of surface tension. The contraction in volume coincident with a decrease in surface tension is scarcely compatible with any other explanation than solvate formation. The decrease in internal pressure is great enough to counteract the decreased solubility due to solvate formation. From 50 to 100 per cent there is little change in the surface tension, but a very marked increase in solubility. The volume is expanding. According to the analysis, this indicates decreasing amounts of solvate.

Conclusion.—Consideration of surface tension, volume, and solubility changes in sulfuric acid and alcohol-water solutions points to the existence of solvates in large amount.

V. Activity of Nitrogen in Protein Solutions.

The solubility of nitrogen in pure protein solutions was determined by means of the apparatus shown in Fig. 1.

1. Preparations.—Blood from a healthy horse was allowed to flow into an iced bottle and immediately defibrinated. After centrifuging in iced containers the plasma was dialyzed in collodion membranes in a refrigerator, the dialyzing flasks being surrounded with ice. The outer liquid in some experiments was distilled water, in others distilled water saturated with CO_2. The dialyzing tubes were stoppered to prevent dilution. Dialysis was continued until no trace of chloride was present in the outer liquid after 12 hours dialysis.

The red cells were washed with 0.8 per cent NaCl three times (by stirring and centrifuging), then an equal volume of distilled water was added with stirring, then saturated NaCl to 2 per cent of the volume to contract the stromata, the mixture centrifuged, and the hemoglobin removed and dialyzed as were the plasma proteins.

In some cases the plasma proteins were extracted with ether before dialysis in order to remove fat, etc., as far as possible. The fat might dissolve N_2 considerably more than water. The fat content of this horse plasma was very low, however. After extraction the dissolved ether was removed by evaporation under the reduced pressure of a water pump. The fat then averaged about 55 mg. per 100 cc. and the cholesterol 30 to 70.

After each experiment with plasma proteins in the second series the specific gravity was determined in a 25 cc. pycnometer, the refractive index with an immersion refractometer, the albumin and globulin by refractometer, and the pH electrometrically. The density and refractometer determinations were done in the large water bath used for the gas experiments, at 20°C. The pH determinations were unsatisfactory, probably on account of the absence of inorganic ions. The readings were variable, and the error may be 0.1 pH either way. In the hemoglobin series and plasma series No. 1, pH and density determinations were omitted. The density and concentration were calculated from the refractometer measurements on the basis of earlier experiments on the relation between concentration from dry weight (drying over $CaCl_2$

at 105°C. in absence of O_2 (by means of N_2)), refractometer measurements, and density.

In the earlier experiments the N_2 gas was bubbled through two large test-tubes of pyro-NaOH to remove traces of O_2. Analysis showed the presence of about 0.3 per cent O_2 which should make no perceptible difference in the figures, but in view of reports of peculiar relations of N_2 solubility to the presence of O_2, the N_2 was collected over pyro-NaOH in a large bottle, and thereafter showed no trace of O_2. The second series of plasma protein experiments done with this N_2 showed a very slightly greater solubility of N_2 instead of less as expected. No difference was made in the hemoglobin solutions.

2. Technique of Experiments.—The advantage of the form of apparatus used is that there are no empirical corrections. It measures the absorption of N_2 by getting the difference in amount in the apparatus before and after shaking with a thoroughly evacuated liquid.

After a satisfactory apparatus had been devised for water, great difficulties were experienced from foaming during extraction in the case of protein solutions, with loss of solution. This was overcome by interposing a large Kjeldahl bulb between the apparatus and the pump, in which the foam collected and from which it was periodically run back into the apparatus by gravity. The evacuation was done with an electric pump capable of a vacuum of 0.01 mm. or less. A large bottle of $CaCl_2$ between the apparatus and the pump absorbed moisture. On account of rapid cooling from evaporation, it was necessary to put an electric stove under the apparatus during evacuation. The temperature never rose above 20°C. The Kjeldahl bulb was kept iced to maintain the vacuum beyond it.

All but one of the stop-cocks concerned in the vacuum about the liquid were mercury-sealed, and the terminal had mercury in the tubing on each side. The exception had mercury run through the bore and had been found by tests to stand a high vacuum. The vapor pressure of the liquid prevented the vacuum from reaching a high figure in the experiments. Generally it was about 3 to 5 mm. At the end of evacuation the bulb containing the liquid was completely filled by drawing in mercury from below while

the vacuum was still on, thus avoiding any possibility of leakage.

After about ¾ of an hour's shaking and evacuation, the maximum gas solubility was obtained with plasma proteins, but hemoglobin took about 1½ hours.

3. Description and Use of Apparatus.—Bulb *C* is filled with mercury drawn up through tube 8 to level 9. Mercury is run

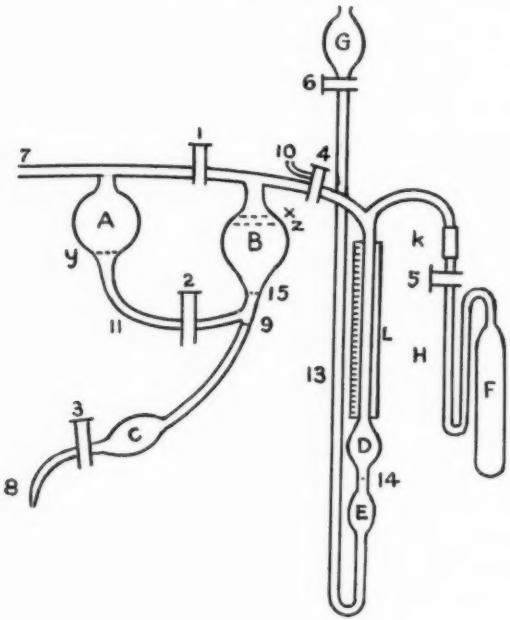

FIG. 1.

through bore of 4 through tube 10. Solution is drawn in through tube 7 and bulb *A* to fill bulb *B* to level *x*. (Bulb *B* holds 63.775 cc. counting space in tubing to stop-cocks.) The solution is allowed to flow back into *A* by gravity, so that both bulbs are about half full, allowing room for shaking. The Kjeldahl bulb and pump are attached at 7. Stop-cock 1 is opened, with 4 and 3 closed. Evacuation and shaking are then started and continued

as above. When completed, the apparatus is tipped up, with the vacuum still on, so that all the solution except enough to fill tube 11 to level y is in bulb B. 2 is then closed. Tip 8 (already full of mercury, and with a protruding drop of mercury put on the tip) is then dipped into a beaker of mercury, and mercury is drawn up until B is full. Then 1 is closed and 3, the pump is detached, and 2 is opened. This relieves all vacuum on the evacuated fluid, which is protected by mercury in 11. Solution from B is then drawn just through 4, and 4 closed.

Tube 10 is then connected with a three-way stop-cock leading to the pump and the gas supply. Liquid in the manometer is drawn to 5. G is half filled with mercury, and 13 to level 14. There is a thin layer of water on top of the mercury. Bulb D and the tubing space to 4 and 5 are then alternately evacuated to about 15 mm. and filled with gas. Evacuation is done by rinsings to avoid any possibility of leakage under high vacuum, but even more to avoid evaporating the film of water on the mercury, which is necessary to saturate the gas with water vapor, although the gas is drawn through a water bottle at 20°C.

4 is then closed, 5 opened, and the mercury adjusted to 14, which is a mark on a tube of about 1 mm. diameter. The apparatus has been in the constant temperature bath since the rinsings with gas began. Readings are now made on manometer H which contains water as the liquid. This enables readings to be made easily to less than 1 mm. without the use of any cathetometer, corresponding to about 0.074 mm. of Hg. There is no appreciable error from solubility of the gas in the water, on account of the small volume of the water (the manometer is 1 mm. tubing) and the fact that the water is nearly saturated to begin with. Diffusion has been shown to be very slow in capillary tubes. The capillarity introduces no error, as the pressure is empirically standardized, and at the end of the experiment the pressure is brought back to the same point. The mercury is run carefully up and down about 14 between each two readings, by means of a Van Slyke pump attached to the top of G, in order to make a fresh contact of the water in the manometer and prevent sticking. The heavy wall rubber connection K introduces no danger of leaks, since it is never subjected to a change of pressure of more than a few mm. of water.

The apparatus is now taken out of the bath, 4 is opened, and

mercury run out of 8 into a weighed flask, while 6 is opened to allow mercury to run at about the same rate into bulb D until about 11.9 cc. of mercury have been run out. This allows space enough in B for shaking.

The apparatus is put in the bath and shaken, and more mercury run into D at intervals, keeping the pressure about atmospheric. After about 30 minutes absorption is slower, and the manometer is opened.

Equilibration is continued until three readings about 10 minutes apart show no change in the pressure. This usually takes from $2\frac{1}{2}$ to 4 hours. The solution in B is stirred very effectively by the mercury at the bottom. Mercury in L is then adjusted to bring the manometer level to the point at which the original gas volume was read. Careful tests showed no change in absorption with the apparatus at rest during these small changes of pressure.

After equilibration, the apparatus is taken out of the bath, and mercury run out of 8 into a weighed flask until level in B reaches a mark 15. The space above this including the tubing to the cocks 1 and 4 through the bore of 4 is calibrated.

4. Calculation.—It is unnecessary to know the total volume of the gas in the apparatus, since the pressure at the end and at the beginning is the same and the temperature is constant. There is no difference of volume in the manometer. The volume change is measured by the change in D and L minus the mercury run out of 8. The apparatus was calibrated with mercury while wet with water, as in the experiments. The calibration was repeated at intervals. The pressure of absorption was calculated from the manometer reading, allowing for the change in pressure in the bulb (the whole manometer including bulb, was calibrated) due to rise or fall in the further limb.

If A = original gas volume,
" B = final " "
" pA = original pressure − *v.p.* of water = final pressure,
" *pabs* = pressure during equilibration − *v.p.* of water,
then

$$\frac{(A - B)(pA \times 273)}{760 \times 293} = \text{volume of gas absorbed at 0°C. and 760 mm.} = C$$

$$\frac{C \times 760}{\text{volume of liquid} \times pabs} = \text{coefficient } B.$$

5. Analysis of Experimental Results.

a. Plasma Proteins. Tables XVIII to XXV.

1. Solubility.—The solubility of N_2 in water at 20°C. averages 1.561 cc. per 100 cc. of water. In Series 1 of plasma proteins it was 1.5168; in Series 2, 1.5156. The average of all experiments was 1.516 per 100 cc. of solution.

In Table XXIII the results are tabulated in various ways. The solubility in the amount of water present in the solution is calculated from the solubility in pure water, and the excess solubility is calculated per gm. of protein present. This figure is necessarily subject to considerable variation, since it depends on a difference in solubilities. In fact the first digit corresponds to the second decimal of the solubility.

The excess solubility per gm. in each series undergoes no significant variation with pH. None of the so called isoelectric preparations was actually so by the end of the experiment, apparently on account of absorption of base from the glass. There was a definite difference of pH, however, between the isoelectric preparations and those which had not been dialyzed against CO_2-saturated water. (Where the preparations were extracted with ether, the pH is higher, probably because of the soft glass funnel used in the ether extraction.)

The excess solubility does, however, undergo a definite decrease after ether extraction, dropping from an average of 0.00618 for non-extracted to 0.00138 for extracted, in one case, and from 0.00762 to −0.009 in the other case, where, however, there was only one experiment with the extracted. It seems perfectly reasonable that this should be the case on account of the well known high solubility of gases in fats and oils. This, moreover, very likely explains Bohr's figures of a solubility in plasma about the same as in water, in spite of the NaCl. The plasmas used in these experiments in this paper had a very low initial fat content (about 250 mg.). Findlay, in experiments on the solubility of H_2 in gelatin solutions, found a solubility of 1.94 in 1.53 per cent gelatin and 1.83 in 5.71 per cent, compared with 1.98 for water. In the latter solution the calculated solubility for the amount of water present is 1.89, thus showing a decrease in

solubility over that due to the water. In these solutions there was probably no fat-like substance present.

Findlay thought that there is a certain amount of adsorption of gas in protein solutions, and recently, Conant has postulated adsorption of nitrogen in hemoglobin solutions in the presence of oxygen. However at atmospheric pressure the adsorbed gas is apparently relatively small compared with the total gas dissolved, so that we probably have only a small error here on account of adsorption. Besides, oxygen is absent, and adsorption is apparently very slight under such conditions. The evidence for adsorption in any case is not very convincing.

In conclusion, the evidence points to a solubility in fat-free plasma protein solutions of 5.8 per cent concentration of about 1.50 cc. per 100 cc. of solution compared with 1.561 for water. This figure is the same as that for the solubility due to the amount of water present, so that there is apparent no excess solubility due to the protein, although an excess solubility would be expected from internal pressure considerations taken up below.

2. Contraction of Volume.—Table XXV gives the volume ratio, calculated from the data of Chick and Martin, for serum albumin and globulin solutions. There is a marked contraction in volume, which could not be accounted for by depolymerization of water, even assuming the protein molecule to be of as low molecular weight as sucrose, for instance.

3. Internal Pressure.—That there is a marked lowering of surface tension in plasma protein solutions seems to be evident from the measurements of various observers, however inaccurate the absolute amount of lowering may be in any individual case. This is corroborated by the tendency of protein to concentrate in surfaces.

Henderson, in experiments on the compressibility of protein solutions, found about the same compressibility as in the case of water. Assuming for the moment that there is some relation between compressibility and cohesive forces between molecules in solution, it is apparent that when we consider these forces for purposes of solubility, we want the force per molecule, not the total force in a certain volume of solution. If the compressibility figures are divided by the mols present, we should get a truer figure, but apparently this change would not be definitely outside

of the errors of the determinations. In the case of extremely large molecules it is probable that the atoms within the molecule attract each other and compress the molecule, but are too far away from the atoms in other molecules to affect them. This would result in decreased compressibility of the molecule but no increase in attractive force between this molecule and another molecule. Probably this internal mass of atoms would attract the atoms on the surface, leaving them less free attractive force to attract other molecules. This is simply an extension of the idea apparently proved earlier in the paper, when it was found that there may occur contraction in volume with marked decrease in internal pressure. The fact that the compressibility is about the same as in the case of water would, then, really indicate that the cohesive forces between molecules are less. Thus this interpretation of compressibility agrees with surface tension deductions in giving the protein molecules low cohesive force. Former methods of interpreting compressibility conflict with the facts of surface tension.

A further piece of evidence for low internal pressure of the proteins is derived from the gas solubility measurements. The previous analysis showed that contraction in volume meant decreased solubility over that due to the mixture law. In the case of plasma protein solutions, the solubility of N_2 is about the same as in the water of the solution, in spite of a contraction in volume; therefore the original solubility in the pure protein must be high (and, of course, very high per mol), because high solubility of an inert gas is characteristic of substances of low internal pressure.

The solubility of the proteins themselves in water is another piece of evidence in the same direction. Cohn has shown that the proteins, when carefully purified, are very insoluble in water, and extremely insoluble when the solubility is figured on a molar basis, which, of course, is the proper method for these purposes. Their internal pressure therefore should be far removed from that of water. We cannot assume that it is greatly higher without contradicting the other evidence, including the lowering of surface tension in solution and the concentration in surfaces. Therefore it must be greatly lower.

4. Question of Hydration.—From the previous analysis contraction in volume means either increased internal pressure or solvate formation. We have no way of telling whether the internal

pressure here is less than it would be on account of simple mixing. But we have one step in the passage from pure water to pure protein by way of protein solutions; and if we examine the situation in the plasma protein solutions compared with water, we see that the solubility per mol (practically the same as the solubility per mol of water in this case) has not changed, while the internal pressure has considerably decreased. At the same time there has been a contraction in volume. By the same reasoning as in the case of the alcohol-water and sulfuric acid-water solutions this should mean solvate formation. The amount of solvate does not appear to vary with such variations of pH as could occur in human blood. The absolute amount of hydration cannot be estimated, but it will be greater in so far as adsorption accounts for part of the gas solubility.

5. Significance of Hydration.—Although hydration is probably present, it does not have the significance usually attached to it in biochemical literature. In the case of substances of low internal pressure as solute, the decreased solubility due to the solvate is counteracted by the low internal pressure of the solution. The net result for the inert gases is the same as if there were a substance added to the water of zero gas solubility and the same internal pressure as the water.

For substances of internal pressure between the gases and the solution, the situation is complicated by the fact that we do not know the amount of solvate, so cannot say how much effect must be allotted to it in comparison to the internal pressure considerations. For most substances the polar groups of the protein will be important and may entirely neutralize the decrease in solubility due to solvation.

In view of the earlier analysis of the factors in activity, it seems entirely unsound to use the solubility of an inert gas as the measure of the activity of any other substances. The activity of each third component in protein solutions will have to be determined separately unless much more accurate methods of calculating solubilities are devised than exist at present.

b. Hemoglobin Solutions.

On account of lack of data as to surface tension and volume change, no discussion is possible of the data on solubility of N_2

in hemoglobin solutions given in Table XX. It may be noted that there is a large excess of solubility per gm. of protein.

SUMMARY.

An analysis is made showing the complexity of the relations of the activity of a third component in a two component solution. Previous attempts to determine hydration from activity measurements are criticized.

An activity ratio is suggested as a means of determining deviations of activity (or solubility) from that due to simple mixing of components. Comparison of this activity ratio with an analogous volume ratio in simple systems consisting of inert gases dissolved in mixtures of organic liquids shows an apparently general rule that deviations in activity vary in accord with deviations in volume. The deviations in volume, however, do not necessarily vary with internal pressure changes. The question of solvation is discussed in connection with these and other factors, and the conclusion reached that solvation is present in alcohol-water and sulfuric acid-water solutions.

Experiments are tabulated concerning the solubility of N_2 in pure plasma protein solutions. The solubility ordinarily obtained is shown to be in part dependent on the fat content. The true solubility is less than would be expected from other considerations. The general situation suggests the presence of solvates, which, however, do not vary with moderate pH differences. The net effect of the solvate formation on the activity of N_2 is zero on account of other factors.

The activity of other substances than N_2 cannot be identified with the activity of N_2, but must be determined in each case separately. The probability is that decreased solubility due to solvates will not be in evidence on account of the low internal pressure and the polar groups of the protein molecule.

Conclusions as to Relationships of Proteins in Solutions and One of Their Functions in Biology.

If the proteins are substances of low internal pressure, but of highly polar nature, with capacity to combine with water, and many other things, their behavior should be governed by the known

effects of such characteristics, which may be antagonistic in some cases and mutually enforcing in others.

Solubilities of Proteins.—On account of their low internal pressure in relation to water one would expect them to be insoluble like the fats. They should be even more insoluble in solutions of higher internal pressure than water, like solutions of inorganic salts. Their polar groups, however, enable them to combine with water and thus attain a certain solubility. This solubility should be greatly reduced on the addition of large amounts of another substance like alcohol which also has polar groups combining with water, unless of course such a substance combines with the protein. The solubility should be increased by dilute salt solution on account of the polar groups combining with the ions, which are themselves in strongly stable solution. This increase in solubility one would expect to continue until the polar groups had been all combined. The addition of further salt should decrease the solubility by raising the internal pressure of the solution to a higher degree, without, of course, causing any further solubility by combination with polar groups, because these have all been satisfied. High degrees of salt may also dehydrate the polar groups, if the salt has a higher power of hydration. Apparently so far the theoretical consequences of the theory fit well the observed facts.

Activities and Solubilities of Other Substances in Protein Solutions.—Remembering that the activity goes in inverse relation to the solubility, in order to simplify matters, the solubility only will be mentioned.

Substances of much lower internal pressure than the proteins, such as N_2, should have a greater solubility in the protein than in water. As we have seen in the experimental results, this greater solubility is exactly offset by the solvation of the protein, so that the effect on the solubility of an inert gas of increasing the concentration of protein in the solution would be to reduce its solubility in proportion to the reduction in water present. If the gas is of a polar nature, its solubility in the protein would be greater, and, depending on the degree of combination with the protein, of course, its solubility in protein solutions would be greater than accounted for by the amount of water present.

Organic substances of low internal pressure close to that of the proteins, like the fats and other lipoids, should be extremely soluble

in the proteins, and their solubility in protein solutions would probably be far in excess of that in water, especially when they are also influenced by the polar groups in the protein molecule. This may explain a great deal of the stabilizing effect of proteins on fat emulsions and the presence of lipoids in protein solutions which are difficult to extract with fat solvents because the proteins themselves are fat solvents. The proteins and lipoids by virtue of their low internal pressure will tend to concentrate in surfaces, where their mutual solubility may be a very important factor in the formation of the cell membrane.

Other organic substances of presumably lower internal pressure than water, such as urea, uric acid, and amino acids, should be more soluble in protein solutions than in water, especially where their polar nature enables them to combine with the protein. This may explain the tendency of such substances to go where the protein concentration is highest—for instance, why the amino acids are absorbed out of the blood into the muscles, or why the concentration of urea in the red cells is about the same as in the plasma, in spite of the greatly reduced water content of the red cells. It may be of significance here that the experiments reported in this paper showed a much greater solubility in hemoglobin solutions than in plasma solutions.

Inorganic salts, however, should be less soluble in protein solutions than in water, except as they combine with the protein. Since most of those present in body fluids do combine to some extent, it is a question whether their solubility when present in the usual amounts may not be the same as in water, or greater.

Thus it seems that the proteins may have a function in changing the solubilities of substances in solution in the body fluids and tissues. The quantitative relations remain to be worked out. Such changes in solubility will affect the distribution of substances, and thus influence absorption, secretion, diffusion, and excretion.

These conclusions seem a reasonable result of the data presented in this paper, and they appear to be in harmony with the other known facts. It would be highly desirable, however, to have confirmation and extension of the data, and until this is done the conclusions can only be regarded as provisional.

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TABLE I.

Glycerol-Water. $\frac{\text{Volume Calculated}}{\text{Volume Observed}}$ *at 20°C.*

From specific gravity $\frac{20°}{4°}$ (Gerlach).

Weight per cent of glycerol.	Glycerol per 100 cc. solution.	H_2O per 100 cc.	Volume calculated / Volume observed.	Glycerol per 100 cc.	H_2O depolymerized.	H_2O depolymerized.	Contraction volume from depolymerization.	Ratio corrected.
	gm.	*gm.*		*mols*	*per cent*	*gm.*	*cc. per 100 cc. solution*	
0			1.0000					
10	10.22	91.95	1.0022	0.111	0.311	0.286	0.0429	1.0017
20			1.0045					
30	32.15	75.06	1.0071	0.349	0.977	0.734	0.1102	1.0061
40			1.0095					
50	56.25	56.25	1.0109	0.6115	1.713	0.9635	0.1456	1.00945
55			1.0112					
60	46.195	46.195	1.0112	0.75	2.10	0.974	0.146	1.00974
65			1.0109					
70	35.391	35.391	1.0102	0.8965	2.51	0.888	0.133	1.0089
80	24.186	24.186	1.0082	1.048	2.93	0.709	0.1065	1.00714
90			1.0050					
95			1.0018					
100			1.0000					

TABLE II.

Glycerol-Water. Activity Ratio of H_2.

a. Drucker's Figures.

Weight per cent of glycerol.	$\frac{D\ 25°}{25°}$	$\frac{D\ 25°}{4°}$	$\lambda 25°$ per 100 cc.	$\lambda 25°$ per 100 gm. solution.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated per 100 gm. solution.	Solubility calculated / Solubility observed.
0.0	1.0000	0.99707	1.96	1.966				1.000
4.0	1.0101	1.00714	1.86	1.847	0.0083	1.886	1.894	1.025
10.5	1.0260	1.02300	1.78	1.739	0.0218	1.760	1.780	1.024
22.0	1.0542	1.0511	1.54	1.465	0.0457	1.533	1.579	1.077
49.8	1.1290	1.1257	0.99	0.879	0.1035	0.987	1.095	1.246
50.5	1.1300	1.1267	0.97	0.862	0.1050	0.973	1.078	1.252
52.6	1.1365	1.13317	0.90	0.794	0.1095	0.932	1.042	1.312
67.0	1.1752	1.1717	0.67	0.572	0.1395	0.649	0.788	1.378
80.0	1.2113	1.2078	0.51	0.422	0.1665	0.393	0.559	1.325
82.0	1.2159	1.21233	0.51	0.420	0 1705	0.353	0.5235	1.247
88.0	1.2307	1.2271	0.44	0.358	0.1830	0.236	0.419	1.170
95.0	1.2502	1.2465	0.34	0.273	0.1975	0.098	0.296	1.083
100.0	1.0000	1.2496	0.26*	0.208				1.000

b. Muller's Figures.

The calculations for this table were made in a slightly different way from the preceding.

Weight per cent of glycerol.	$\frac{D\ 15°}{4°}$	Glycerol per 100 cc. solution.	B observed at 15° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated per 100 cc.	Solubility calculated / Solubility observed.
		gm.					
0.0	0.99913	0.00	1.892				1.000
14.9	1.0353	15.42	1.647	0.1135	1.67	1.78	1.08
22.8	1.0526	24.02	1.510	0.1768	1.54	1.714	1.135
38.0	1.0945	41.59	1.216	0.306	1.287	1.59	1.307
43 5	1.1115	48.50	1.110	0.356	1.188	1.54	1.522
49.15	1.1256	55.35	1.010	0.407	1.085	1.49	1.475
51.5	1.1321	58.30	1.025	0.429	1.04	1.47	1.435
68.0	1.1738	79.82	0.806	0.587	0.712	1.30	1.61
90.7	1.2479	113.20	0.870	0.832	0.22	1.05	1.207
100.0	1.2634	126.34	0.93*				1.00

* By extrapolation.

TABLE III.

N_2 in Glycerol-Water Mixtures.

a. Drucker's Figures.

Weight per cent of glycerol.	$D\frac{25°}{4°}$	Glycerol per 100 cc. solution.	λ 25° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated per 100 cc. solution.	Solubility calculated / Solubility observed*
		gm.					
0.0	0.997	0.00	1.56				1.000
16.0	1.0362	16.57	1.03	0.0377	1.36	1.398	1.358
29.7	1.0714	31.80	0.67	0.0723	1.175	1.247	1.86
48.9	1.1233	54.90	0.52	0.125	0.896	1.02	1.96
74.5	1.190	88.70	0.25	0.202	0.473	0.675	2.7
84.1	1.2183	102.50	0.24	0.233	0.301	0.5345	2.23
100.0	1.2496	124.96	0.28*				1.00

b. Muller's Figures.

Weight per cent of glycerol.	$D\frac{15°}{15°}$†	Glycerol per 100 cc. solution.	B 15° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed*
		gm.					
0.00		0.00	1.694				1.000
25.00	1.0620	26.55	1.266	0.193	1.356	1.55	1.223
42.2	1.1079	46.80	0.976	0.34	1.088	1.43	1.465
51.5	1.1332	58.35	0.759	0.424	0.934	1.36	1.79
58.0	1.1514	66.80	0.703	0.4855	0.822	1.308	1.86
80.25	1.2132	97.40	0.530	0.60	0.406	1.006	1.897
90.0	1.2395	111.50	0.583	0.687	0.212	0.899	1.70
95.0	1.2525	119.00	0.716	0.80	0.106	0.906	1.265
100.0			0.894*				

TABLE III—*Concluded.*

c. von Hammel's Figures.

Weight per cent of glycerol.	$D\frac{15°}{15°}$	Glycerol per 100 cc. solution.	B 15° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed
		gm.					
0.00	1.00	0.00	1.707				1.000
15.7	1.0382	16.31	1.425	0.0688	1.493	1.562	1.095
29.9	1.0747	32.1	1.087	0.1357	1.287	1.423	1.308
46.6	1.1198	52.15	0.84	0.22	1.02	1.24	1.475
57.6	1.1531	66.45	0.698	0.28	0.834	1.114	1.595
67.1	1.1774	79.0	0.635	0.333	0.66	0.99	1.56
72.8	1.1931	86.9	0.552	0.367	0.551	0.918	1.665
74.7	1.1983	89.5	0.597	0.378	0.518	0.896	1.50
77.0	1.2044	92.45	0.527	0.391	0.478	0.869	1.65
85.1	1.2263	104.5	0.482	0.441	0.309	0.75	1.555
87.3	1.2323	107.7	0.492	0.454	0.265	0.719	1.46
88.5	1.2354	109.5	0.536	0.462	0.239	0.701	1.305
99.25	1.2631	125.5	0.524	0.529	0.0138	0.543	1.035
100.0			0.523*				1.000

* By extrapolation.

† The correction to $D\frac{15°}{4°}$ amounts to so little in comparison with experimental errors that it was not considered worth while to make it.

TABLE IV.

O_2 in Glycerol-Water Mixtures.

Figures from Muller.

Weight per cent of glycerol.	$D\ \frac{15°}{15°}$	Glycerol per 100 cc. solution.	B 15° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
		gm.					
0.00	1.000	0.00	3.400				1.000
20.5	1.0503	21.53	2.742	0.150	2.805	2.955	1.077
25.0	1.0620	26.55	2.521	0.186	2.710	2.90	1.15
37.3	1.0947	40.80	2.022	0.286	2.335	2.62	1.295
45.0	1.1155	50.20	1.744	0.353	2.085	2.44	1.40
52.0	1.1346	59.00	1.570	0.413	1.850	2.26	1.44
71.5	1.1895	85.00	0.950	0.595	1.550	2.14	2.25
88.5	1.2354	109.50	0.886	0.766	0.477	1.24	1.40
100.0			0 886*				1.00

* By extrapolation.

TABLE V.

CO_2 in Glycerol-Water Mixtures.

Figures from von Hammel.

Weight per cent of glycerol.	$D\ \frac{15°}{15°}$	Glycerol per 100 cc. solution.	B 15° per 100 cc.	Solubility calculated in glycerol.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
		gm.					
0.00	1.000	0.00	100.3				1.000
26.11	1.0649	27.8	78.5	9.0	78.9	87.9	1.12
27.69	1.069	29.6	80.0	9.58	77.5	87.1	1.09
43.72	1.112	48.6	63.9	15.75	62.8	78.5	1.23
46.59	1.1198	52.05	62.0	16.9	61.0	77.9	1.257
62.14	1.1632	72.30	51.1	23.4	44.1	67.5	1.32
73.36	1.1946	87.60	44.9	28.4	31.96	60.36	1.345
77.75	1.2064	93.80	43.0	30.4	26.94	57.34	1.335
87.74	1 2334	108.30	42.2	35.1	15.09	50.2	1.19
90.75	1.2415	112.70	40.4	36.5	11.5	48.0	1.19
96.64	1.2566	121.50	41.5	39.4	4.2	43.6	1.05
99.26	1.2631	125.5	41.0	40.66	0.8	41.46	1.01
100.00	1.265	126.3	41.0				1.000

TABLE VI.

CO in Acetic Acid-Benzene Mixtures.

Densities from Hubbard. Gas solubility from Skirrow.

a. Volume Change.

Weight per cent of acetic acid.	Volume at 25°.	Calculated volume of acetic acid.	Calculated volume of benzene.	Total volume calculated.	Volume calculated / Volume observed.
0.00	1.1446				1.0000
11.634	1.1277	11.154	101.14	112.294	0.99578
23.60	1.1092	22.206	87.447	110.053	0.99175
34.0	1.0919	32.569	75.543	108.112	0.99013
63.97	1.0369	61.276	41.24	102.516	0.98868
73.82	1.0169	70.711	29.966	100.677	0.99004
100.00	0.9579				1.00000

b. Gas Activity.

Weight per cent of acetic acid.	Volume at 25°.	λ 25° per cc. solution.	λ 25° per gm. solution.	λ in acetic acid.	λ in benzene.	λ calculated, total.	Solubility calculated / Solubility observed.
0.00	1.1446	0.174	0.199				1.000
19.17	1.1156	0.190	0.212	0.0316	0.16	0.1916	0.903
33.54	1.0932	0.198	0.216	0.0553	0.1322	0.1875	0.866
67.51	1.0292	0.199	0.205	0.1112	0.065	0.1762	0.859
100.00	0.9579	0.172	0.165				1.000

TABLE VII.

CO in Acetone-Carbon Disulfide Mixtures.

Data sources as in Table VI.

a. Volume Change.

Weight per cent of CS_2.	Volume at 25°.	Calculated volume of CS_2.	Calculated volume of acetone.	Total volume calculated.	Volume calculated / Volume observed.
0.00	1.26905				1.00000
13.245	1.21357	10.5458	110.096	120.6418	0.99411
29.326	1.14319	23.3496	89.6888	113.0384	0.98879
40.329	1.09304	32.1103	75.7255	107.8358	0.98657
51.761	1.03962	41.2126	61.2177	102.4303	0.985264
51.799	1.03937	41.2429	61.1695	102.4124	0.985328
51.902	1.03847	41.3249	61.0388	102.3637	0.98572
64.733	0.97694	51.5411	44.7556	96.2967	0.98571
71.137	0.94547	56.640	36.6286	93.2686	0.986479
83.283	0.88407	66.3109	21.2147	87.5257	0.99003
86.892	0.86551	69.1843	16.6347	85.8190	0.991543
100.000	0.79621				1.000000

b. Gas Activity.

Weight per cent of CS_2.	Volume at 25°.	λ 25° per cc. solution.	λ 25° per gm. solution.	λ in CS_2.	λ in acetone.	λ calculated, total.	Solubility calculated / Solubility observed.
0.00	1.26905	0.238	0.30202				1.000
8.18	1.2330	0.236	0.291	0.00624	0.2773	0.2835	0.9743
18.02	1.1925	0.236	0.2814	0.01376	0.2476	0.2614	0.9287
49.46	1.0500	0.227	0.2384	0.03776	0.1526	0.1904	0.7988
62.60	0.987	0.210	0.2073	0.0478	0.11295	0.16075	0.7757
74.05	0.941	0.187	0.176	0.0565	0.0784	0.1349	0.7667
85.51	0.873	0.144	0.1257	0.0653	0.0438	0.10905	0.8675
96.42	0.815	0.114	0.0929	0.0736	0.0108	0.0844	0.9088
100.00	0.79621	0.0959	0.0764				1.0000

TABLE VIII.

CO in Acetone-Chloroform Mixtures.

Data as in Table VII.

a. Volume Change.

Weight per cent of $CHCl_3$.	Volume at 25°.	Calculated volume of $CHCl_3$.	Calculated volume of acetone.	Total volume calculated.	Volume calculated / Volume observed.
0.00	1.274				1.000
16.66	1.1744	11.257	106.17	117.427	0.9999
33.04	1.0764	22.325	85.306	107.631	0.9999
45.83	0.9995	30.967	69.013	99.98	1.0003
56.30	0.9364	38.042	55.674	93.716	1.0008
64.50	0.8868	43.582	45.227	88.809	1.0014
72.276	0.83976	48.839	35.310	84.149	1.0021
79.88	0.7941	53.975	25.633	79.608	1.0025
88.41	0.7435	59.738	14.765	74.503	1.0021
94.30	0.7089	63.718	7.2616	70.979	1.0013
100.00	0.6757				1.0000

b. Gas Activity.

Weight per cent of $CHCl_3$.	Volume at 25°.	λ 25° per cc. solution.	λ 25° per gm. solution.	λ in $CHCl_3$.	λ in acetone.	λ calculated, total.	Solubility calculated / Solubility observed.
0.00	1.274	0.238	0.3032				1.000
33.38	1.073	0.226	0.2425	0.0467	0.2020	0.2487	1.0255
53.20	0.955	0.219	0.2091	0.0744	0.1419	0.2163	1.0343
65.03	0.883	0.220	0.1943	0.09096	0.106	0.197	1.014
73.46	0.832	0.212	0.1764	0.10275	0.0805	0.1832	1.039
79.83	0.793	0.204	0.1618	0.1117	0.0612	0.1729	1.068
87.30	0.749	0.207	0.1550	0.1221	0.0385	0.1606	1.036
94.40	0.708	0.205	0.1451	0.1320	0.017	0.149	1.027
100.00	0.6757	0.207	0.1399				1.000

TABLE IX.

H_2 in Alcohol-Water Mixtures

a. Volume Change.

Weight per cent of alcohol.	$D\frac{20°}{4°}$	Alcohol per 100 cc.	Calculated volume of alcohol.	Calculated volume of H_2O.	Volume calculated / Volume observed.
		gm.			
0.00	0.99823				100.0000
5.00	0.98938	4.947	6.2673	94.1577	100.427
10.0	0.98187	9.819	12.4395	88.5247	100.9642
20.0	0.96864	19.373	24.5433	77.6284	102.1717
30.0	0.95382	28.6146	36.2513	66.8854	103.1367
40.0	0.93518	37.4072	47.3905	56.2105	103.6010
50.0	0.91384	45.692	57.8863	45.7728	103.6591
60.0	0.89113	53.4678	67.7374	35.7081	103.4455
70.0	0.86766	60.7362	76.9456	26.0761	103.0217
80.0	0.84344	67.4752	85.483	16.8989	102.392
90.0	0.81797	73.6166	93.2635	8.1945	101.4547
100.0	0.78934	78.934			100.0000

b. Gas Solubility.

Figures from Lubarsch.

Weight per cent of alcohol.	$D\frac{20°}{4°}$	Alcohol per 100 cc.	λ 20° per 100 cc.	Solubility calculated in alcohol.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
		gm.					
0.00			1.93				1.000
9.09	0.98318	8.93	1.43	0.81	1.725	2.535	1.773
16.67	0.97301	16.22	1.29	1.47	1.565	3.035	2.354
23.08	0.96443	22.25	1.17	2.02	1.43	3.45	2.95
28.57	0.95718	27.33	1.04	2.48	1.32	3.80	3.65
33.33	0.9480	31.60	1.17	2.865	1.22	4.08	3.49
50.00	0.91384	45.70	2.02	4.15	0.882	5.03	2.49
66.67	0.87556	58.35	2.55	5.29	0.564	5.85	2.305
100.00			7.15*				1.000

* From Christoff's figures.

TABLE X.

CO in Alcohol-Water Mixtures.

Figures from Lubarsch.

Weight per cent of alcohol.	λ per 100 cc.	Solubility calculated in alcohol.	Solubility calculated in H_2O.	Total solubility calculated.	$\frac{\text{Solubility calculated}}{\text{Solubility observed}}$.
0.00	2.41				1.000
9.09	1.87	2.15	2.15	4.3	2.3
16.67	1.75	3.91	1.955	5.865	3.350
23.08	1.68	5.46	1.786	7.25	4.32
28.57	1.50	6.59	1.646	8.236	5.49
33.33	1.94	7.62	1.523	9.14	4.71
50.00	3.20	10.78	1.10	11.90	3.72
100.00	19.01*				1.000

* From Just.

"$D\ \frac{20°}{4°}$" and "Alcohol per 100 cc." as in Table IX.

TABLE XI.

N_2 in H_2SO_4-Water Mixtures.

a. Volume Change.

Figures from Lunge and Isler for $\frac{15°}{4°}$ corrected to $\frac{21°}{4°}$ by table of Domke in Landolt-Börnstein.

Gm. per liter H_2SO_4.	$D\frac{21°}{4°}$	H_2O per liter.	Volume of H_2SO_4.	Volume of H_2O.	Volume calculated / Volume observed.
		gm.			
0.00	0.99802				1000.00
240.5	1.1472	906.7	131.457	908.29	1039.747
436.7	1.2565	819.8	238.699	821.234	1059.933
525.2	1.3037	778.5	287.073	779.865	1066.938
996.0	1.5448	548.8	544.421	549.762	1094.183
1216.0	1.6499	433.9	664.653	434.661	1099.314
1453.0	1.7541	301.1	794.206	301.618	1095.824
1684.0	1.82345	139.4	920.47	139.644	1060.114
1756.0	1.8336	77.6	959.825	77.736	1037.561
1829.5	1.8295				1000.000

b. Gas Activity.

Figures from Bohr.

Weight per cent of H_2SO_4.	$D\frac{21°}{4°}$	B 21° per 100 cc.	B 21° per 100 gm. solution.	Solubility in H_2SO_4.	Solubility in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.00	As in (*a*) for corresponding gm. per liter which is on corresponding line.	1.56	1.56				1.0000
20.98		0.91	0.794	0.2111	1.234	1.445	1.82
34.75		0.72	0.569	0.3498	1.018	1.368	2.403
40.30		0.66	0.506	0.4055	0.9305	1.336	2.64
64.50		0.49	0.317	0.649	0.554	1.203	3.795
73.80		0.48	0.292	0.7425	0.409	1.152	3.945
82.80		0.51	0.291	0.833	0.268	1.101	3.785
92.35		1.00	0.548	0.929	0.1194	1.048	1.914
95.80		1.29	0.703		0.0655	1.0295	1.465
100.00		1.84*	1.006				1.000

TABLE XI—*Concluded.*

c. Gas Activity.

Figures from Christoff.

Weight per cent of H_2SO_4.	$D\frac{20°}{4°}$	λ per 100 cc. at 20°C.	λ per 100 gm.	Solubility calculated in H_2SO_4.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.000		1.537					1.000
35.82	1.2669	0.8447	0.667	0.467	0.986	1.453	2.18
61.62	1.5268	0.6144	0.4025	0.804	0.59	1.394	3.46
95.60	1.8348	1.6720	0.912	1.246	0.0676	1.3136	1.44
100.00	1.8305	2.38†	1.303				1.000

* By extrapolation.

† By extrapolation from solubility curve.

TABLE XII.

H_2 in H_2SO_4-Water Mixtures.

Figures from Christoff.

Weight per cent of H_2SO_4.	$D\frac{20°}{4°}$	λ per 100 cc.	Solubility per 100 gm.	Solubility calculated in H_2SO_4.	Solubility calculated in H_2O.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.00		2.077					1.0000
35.82	1.2669	0.9544	0.754	0.258	1.333	1.591	2.11
61.62	1.5268	0.7081	0.464	0.444	0.797	1.241	2.675
95.60	1.8348	1.097	0.598	0.689	0.0914	0.7804	1.305
100.00		1 320*	0.721				1.000

* By extrapolation.

TABLE XIII.

CO in Ethyl Alcohol-Benzene Mixtures.

a. Volume Change.

Figures from Polowzow.

Weight per cent of alcohol.	D 20°.	Alcohol per liter.	Volume of alcohol calculated.	Volume of benzene calculated.	Total volume calculated / Volume observed.
		gm.			
0.00	0.8784				
1.772	0.87635	1.553	1.9666	97.997	99.9636
10.94	0.8667	9.48	12.004	87.875	99.879
23.22	0.8629	20.036	25.372	75.42	100.792
27.81	0.85173	23.69	30.00	69.996	99.996
57.31	0.8262	47.35	59.96	40.154	1001.13
100.00	0.7897				

b. Gas Activity.

Figures from Skirrow.

Weight per cent of alcohol.	D 20°.	λ 25° per cc.	λ per gm.	Solubility calculated in alcohol.	Solubility calculated in benzene.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.00	0.8784	0.174	0.198				1.000
15.43	0.8628	0.179	0.2075	0.0375	0.1674	0.205	0.988
52.34	0.8300	0.181	0.2181	0.1272	0.09437	0.2216	1.016
100.00	0.7897	0.192	0.2431				1.000

TABLE XIV.

CO in Aniline-Acetone Mixtures.

a. Volume Change.

Data from Faust.

Weight per cent of acetone.	Volume at 18°.	Calculated volume of acetone.	Calculated volume of aniline.	Total volume calculated.	Volume calculated / Volume observed
0.00					1.0000
21.092	102.3	26.04	77.283	103.323	1.0091
59.27	112.86	73.17	39.892	113.062	1.0018
100.00					1.0000

b. Gas Solubility.

Data from Skirrow.

Weight per cent of aniline.	D 18°.	λ per cc.	λ per gm.	Calculated solubility in aniline.	Calculated solubility in acetone.	Total solubility calculated.	Solubility calculated / Solubility observed
0.00	0.8100	0.238	0.294				1.000
20.83	0.8492	0.179	0.2108	0.0108	0.2326	0.2434	1.155
55.10	0.920	0.110	0.1196	0.0286	0.1319	0.1605	1.343
100.00	1.021	0.053	0.052				1.000

TABLE XV.

CO in Acetic Acid-Toluene Mixtures.

a. Volume Change.

Data from Humburg.

Weight per cent of acetic acid.	D 16°.	Volume of solution.	Volume of acetic acid.	Volume of toluene.	Total volume calculated.	Volume calculated / Volume observed.
0.00	0.8694					1.000
9.6055	0.8813	113.468	9.0987	104.213	111.3117	0.9969
27.446	0.9072	110.229	25.999	83.453	109.452	0.9929
38.492	0.9248	108.131	36.461	70.748	107.2088	0.9915
100.000	1.0557					1.0000

b. Gas Activity.

Data from Skirrow.

Weight per cent of acetic acid.	D 16°.	λ 25° per cc.	λ per gm. of solution.	Solubility in acetic acid.	Solubility in toluene.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.00	0.8694	0.182	0.2093				1.000
20.48	0.898	0.190	0.2116	0.0336	0.1665	0.2001	0.9457
56.89	0.9484	0.195	0.2056	0.09268	0.09025	0.1829	0.8895
74.71	0.972	0.191	0.19605	0.1217	0.0529	0.1746	0.8906
100.00	1.0557	0.172	0.1629				1.0000

TABLE XVI.

CO in Toluene-Nitrobenzene.

a. Volume Change.

Data from Philip.

Weight per cent of toluene.	D 16°.	Volume of solution.	Volume of toluene.	Volume of nitrobenzene.	Total volume calculated.	Volume calculated / Volume observed.
0.00	1.207					1.000
41.896	1.036	0.9652	0.48156	0.4814	0.96296	0.9976
64.282	0.963	1.0384	0.7388	0.2959	1.035	0.9964
81.789	0.913	1.0953	0.9401	0.15088	1.091	0.9961
100.000	0.870					1.0000

b. Gas Activity.

Data from Skirrow.

Weight per cent of toluene.	D 16°.	λ 25° per cc.	λ per gm.	Solubility in toluene.	Solubility in nitrobenzene.	Total solubility calculated.	Solubility calculated / Solubility observed.
0.00	1.207	0.093	0.07705				1.0000
23.69	1.112	0.108	0.0971	0.0496	0.0588	0.1084	1.116
50.92	1.006	0.131	0.1302	0.1065	0.0378	0.1443	1.108
73.21	0.940	0.151	0.1606	0.15315	0.0206	0.1738	1.1082
81.77	0.914	0.160	0.17505	0.17105	0.01405	0.185	1.0574
91.14	0.891	0.168	0.18855	0.19066	0.00683	0.1975	1.0473
100.00	0.870	0.182	0.2092				1.000

TABLE XVII.

CO in Benzene-Nitrobenzene.

a. Volume Change.

Data from Philip.

Weight per cent of benzene.	D 16°.	Volume of solution.	Volume of benzene.	Volume of nitrobenzene.	Total volume calculated.	Volume calculated / Volume observed
0.00	1.207					1.0000
26.828	1.096	91.24	30.412	60.62	91.034	0.9977
42.28	1.043	95.88	47.93	47.82	95.75	0.9986
75.22	0.942	106.15	85.27	20.53	105.80	0.9967
100.00	0.8821					1.0000

b. Gas Activity.

Data from Skirrow.

Weight per cent of benzene.	D 16°.	λ 25° per cc.	λ per gm.	Solubility in benzene.	Solubility in nitrobenzene.	Total solubility calculated.	Solubility calculated / Solubility observed
0.00	1.207	0.093	0.07705				1.0000
16.73	1.135	0.1015	0.0894	0.033	0.06416	0.0972	1.087
45.1	1.036	0.1265	0.1221	0.08896	0.0423	0.1313	1.075
59.39	0.989	0.140	0.14155	0.1171	0.0313	0.148	1.0483
71.84	0.954	0.152	0.1593	0.1417	0.0217	0.1634	1.0255
85.69	0.894	0.162	0.1812	0.16903	0.011025	0.18005	0.9936
100.00	0.882	0.174	0.1973				1.0000

XUM

TABLE XVIII.

Solubility of N_2 in Water with Earlier Form of Apparatus Impractical for Protein Solutions. All Experiments in Series Included.

	B 20° per 100 cc.
	1.565
	1.564
	1.572
	1.53
	1.53
	1.552
	1.559
	1.563
	1.562
Average.	1.555

TABLE XIX.

Solubility of N_2 in Water with Later Form of Apparatus. All Experiments in Series Tabulated.

Series 1. B 20° per 100 cc.	Series 2. B 20° per 100 cc.
1.536	1.566
1.563	1.582
1.560	1.543
1.558	1.541
1.616	1.554
1.57	1.523 N. B. Short evacuation.
1.557	1.551
Average.....1.565	1.557 omitting value 1.523, probably erroneous.

General average of above averages. 1.561

TABLE XX.

Solubility of N_2 in Hemoglobin Solutions. All Experiments in Series Given.

Experiment No.	Preparation.	Concentration.*	Density.†	H_2O per 100 cc.	Solubility in H_2O of solution, calculated.	Solubility (B) per 100 cc. solution, found.	Excess solubility per 100 cc. solution.	Excess per gm. of Hb.	Excess per mol of Hb.
1	Isoelectric.	9.62	1.02278	92.658	1.446	1.625	0.179		
2	"	9.68	1.0229	92.613	1.446	1.594	0.148		
3	"	9.31	1.0220	92.889	1.450	1.570	0.120		
4	"	9.38	1.0219	92.812	1.449	1.6225	0.1735		
5	"	9.38	1.0219	92.812	1.449	1.633	0.184		
6	Base.	9.06	1.02135	93.075	1.453	1.625	0.172		
7	"	8.56	1.02008	93.448	1.459	1.621	0.162		
Average.....		9.28		92.90	1.4503	1.613	0.163	0.0176	281.0

* By refractometer.

† By calculation from concentration on basis of studies of relation between density, refractive index, and concentration from dry weight; represents $\frac{20°}{4°}$.

TABLE XXI.

Solubility of N_2 in Plasma Protein Solutions. Series 1. All Experiments Included.

Experiment No.	Preparation.	Total protein* per 100 cc. solution.	Albumin.	Globulin.	pH.	Density†	H_2O per 100 cc.	Solubility in H_2O of solution, calculated.	Solubility (B) per 100 cc. solution, found.	Excess solubility per 100 cc. solution.	Excess per gm. of protein.
1	Isoelectric.	5.80	2.72	3.08		1.0145	95.653		1.532		
2	"	5.76	2.90	2.86	5.8	1.0144	95.68		1.5266		
3	"	5.84	3.05	2.79		1.01465	95.625		1.497		
4	"	5.84	3.05	2.79		1.0165	95.81		1.516		
5	Base.	6.32	4.66	1.66		1.01599	95.28		1.511		
6	"	6.69	5.44	1.25	6.3	1.017	95.01		1.514		
7	"	6.66	5.22	1.44	6.4	1.01695	95.035		1.517		
8	Isoelectric.	5.62	4.56	1.06	5.8	1.014	95.78		1.521		
Average....		6.066	3.95	2.116			95.484	1.4904	1.5168	0.0264	0.00435

* By refractometer.

† As in Table XX.

TABLE XXII.

Solubility of N_2 in Plasma Proteins. Series 2. No Experiments in Series Omitted.

Experiment No.	Preparation.	Total protein.*	Albumin.	Globulin.	pH	Density.†	H_2O per 100 cc.	Solubility in H_2O of solution, calculated.	Solubility (B) per 100 cc. solution, found.	Excess solubility.	Excess per gm. of protein.
1	Base.	6.06	3.57	2.49	7.54	1.01517	95.457		1.512		
2	"	6.22	4.20	2.02	7.44	1.01528	95.308		1.521		
3	"	6.35	4.36	1.99	7.48	1.01551	95.20		1.519		
4	"	6 70	4.41	2.29	7.34	1.01659	94.959		1.544		
5	Isoelectric.	3.59	1.83	1.76	6.17	1.00857	97.267		1.548		
6	Base.	6.21	4.25	1.98	7.29	1.01527	95.317		1.535		
7	Isoelectric, extracted.	5.70	4.43	1.27	7.63	1.0153	95.83		1.441		
8	Base, extracted.	5.54	3.12	2.42	8.10	1.0143	95.89		1.519		
9	" "	6.05	3.17	2.88	8.1	1.01592	95.54		1.485		
10	Isoelectric.	6.64	4.61	2.03	7.64	1.01663	95.023		1.532		
Average....		5.906	3.80	2.11			95.579	1.492	1.5156	0.0236	0.004

* By refractometer.

† By direct determination with 25 cc. pycnometer at 20°, calculated for $\frac{20°}{4°}$.

TABLE XXIII.

Averages of Solubility Determinations on Protein Solutions.

No. of experiments.	Preparation.	Concentration per 100 cc.	H_2O per 100 cc.	Solubility in H_2O of solution.	Solubility (B) per 100 cc. solution.	Excess solubility.	Excess solubility per gm.	Additional notes on preparations.
7	Hemoglobin.	9.284	92.90	1.45	1.613	0.163	0.0176	All experiments.
18	Plasma protein.	5.972	95.54	1.491	1.516	0.0248	0.00415	" "
5	" "	5.772	95.709	1.494	1.5185	0.0245	0.00425	Isoelectric of Series 1.
3	" "	6.56	95.108	1.485	1.514	0.029	0.00442	Base of Series 1.
2	" "	5.115	96.145	1.501	1.54	0.039	0.00762	Isoelectric of Series 2, not extracted.
5	" "	6.308	95.248	1.487	1.526	0.039	0.00618	Base of Series 2, not extracted.
2	" "	5.795	95.715	1.494	1.502	0.008	0.00138	Base of Series 2, extracted.
1	" "	5.70	95.583	1.496	1.441	−0.055	−0.009	Isoelectric of Series 2, extracted.
8	" "	6.401	95.196	1.486	1.522	0.036	0.0056	All with base, not extracted.
7	" "	5.580	95.824	1.496	1.525	0.0286	0.0051	All isoelectric, not extracted.

TABLE XXIV.

Solubility and Relative Activity of N_2 in Protein Solutions Compared with Water.

Preparation.	$\frac{\text{Activity in solution}}{\text{Activity in water}}$.	$\frac{\text{Activity in solution}}{\text{Activity calculated for water of solution}}$.	Mols solution.	Solubility per mol.
Average of Hb	0.968	0.8995		
" " plasma protein	1.029	0.982	5.31	0.285
" " " plus base extracted	0.962	1.005		0.283
Water			5.55	0.281

TABLE XXV.

Contraction in Volume of Protein Solutions.

From data of Chick and Martin.

Substance.	D dry.	D in solution.	Per cent by weight.	Gm. per 100 cc.	Calculated volume of protein.	Volume of H_2O.	$\frac{\text{Total volume calculated}}{\text{Volume observed}}$.
Serum albumin.	1.275	1.0647	22.15	23.58	18.496	83.13	101.826
" globulin.	1.279	1.0428	15.33	15.985	12.498	88.56	101.058
" "	1.312	1.0316	11.05	11.399	8.69	92.033	100.721
" "	1.289	1.0466	16.35	17.112	13.275	87.81	101.085

XUM

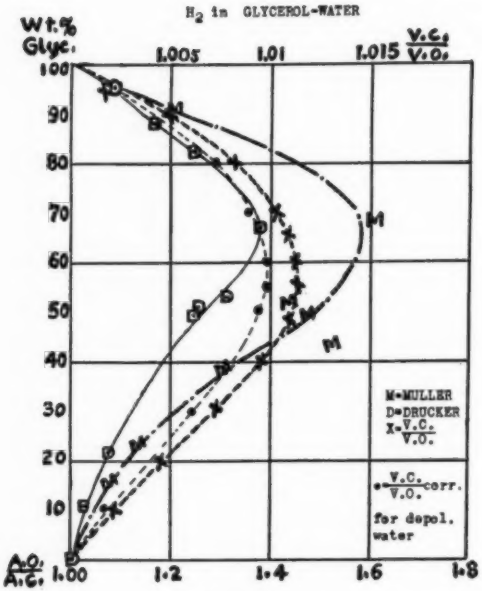
H2 in GLYCEROL-WATER
Wt.% Glyc.
V.C./V.O.
1.005
1.01
1.015
100
90
80
70
60
50
40
30
20
10
A.O./A.C.
1.00
1.2
1.4
1.6
1.8
M
M
M
M-MULLER
D-DRUCKER
X-V.C./V.O.
•-V.C./V.O. corr. for depol. water

CHART I.

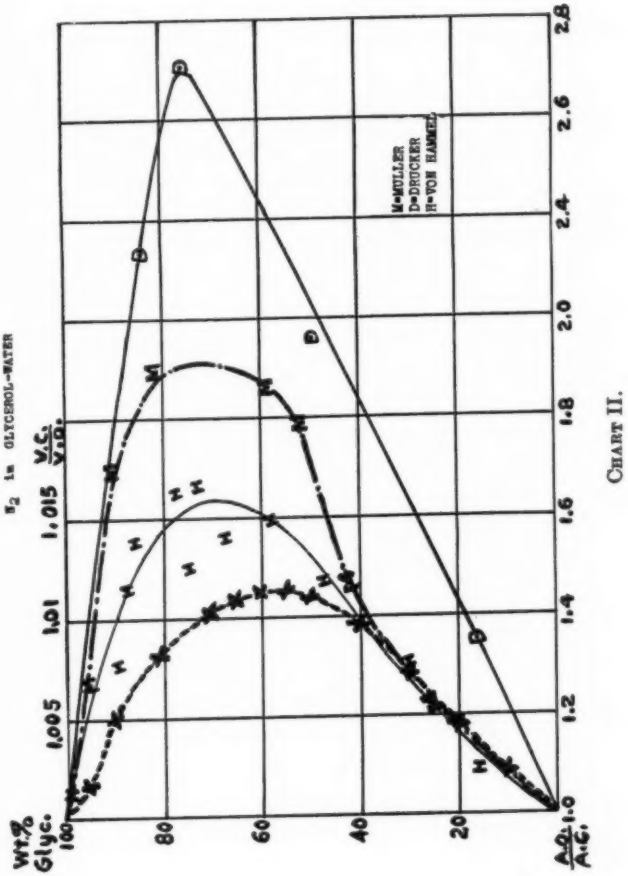
M=MÜLLER
D=DRUCKER
H=VON HANNEL
Wt.% Glyc.
100
80
60
40
20
1.005
1.01
1.015
V.C. V.0.
A.0. / A.C.
1.0
1.2
1.4
1.6
1.8
2.0
2.4
2.6
2.8

CHART II.

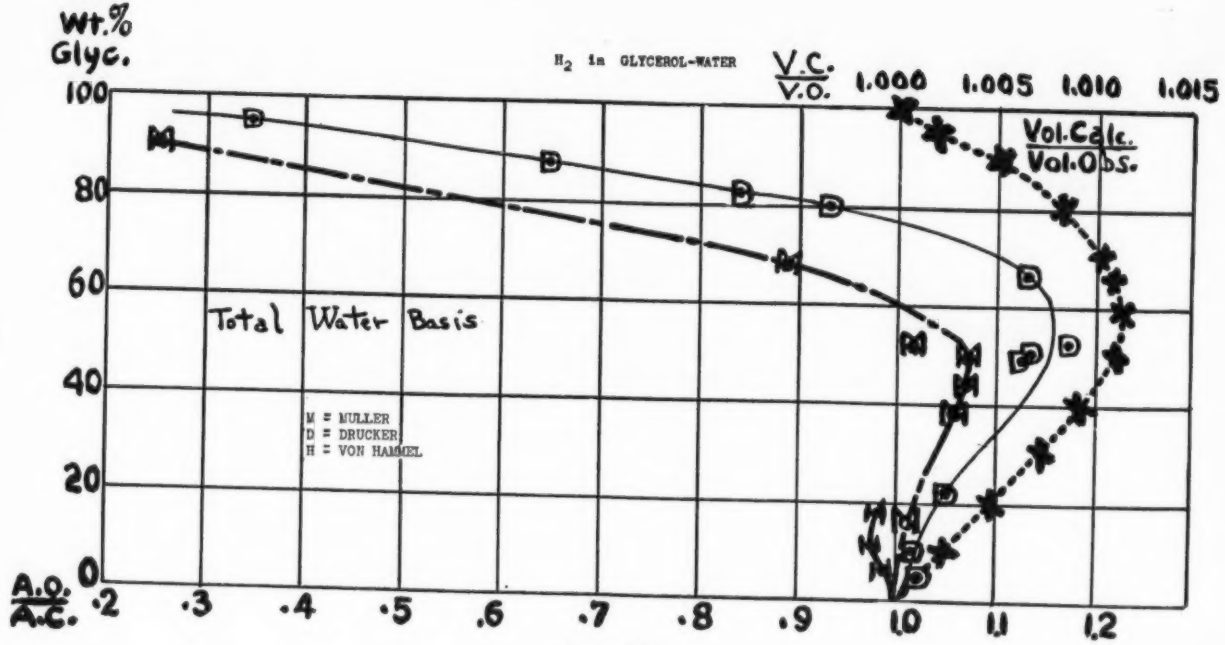
Wt.% Glyc.
H_2 in GLYCEROL-WATER
V.C./V.O.
1.000
1.005
1.010
1.015
Vol.Calc./Vol.Obs.
100
80
60
40
20
0
Total Water Basis
M = MULLER
D = DRUCKER
H = VON HAMMEL
A.O./A.C.
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2

CHART III.

CHART III.

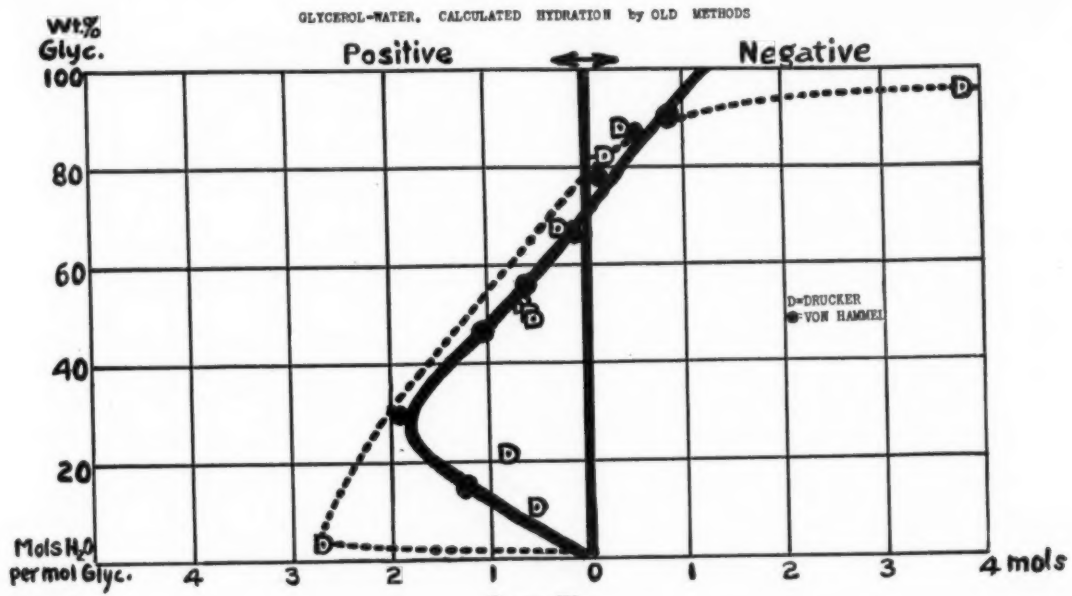

CHART IV.

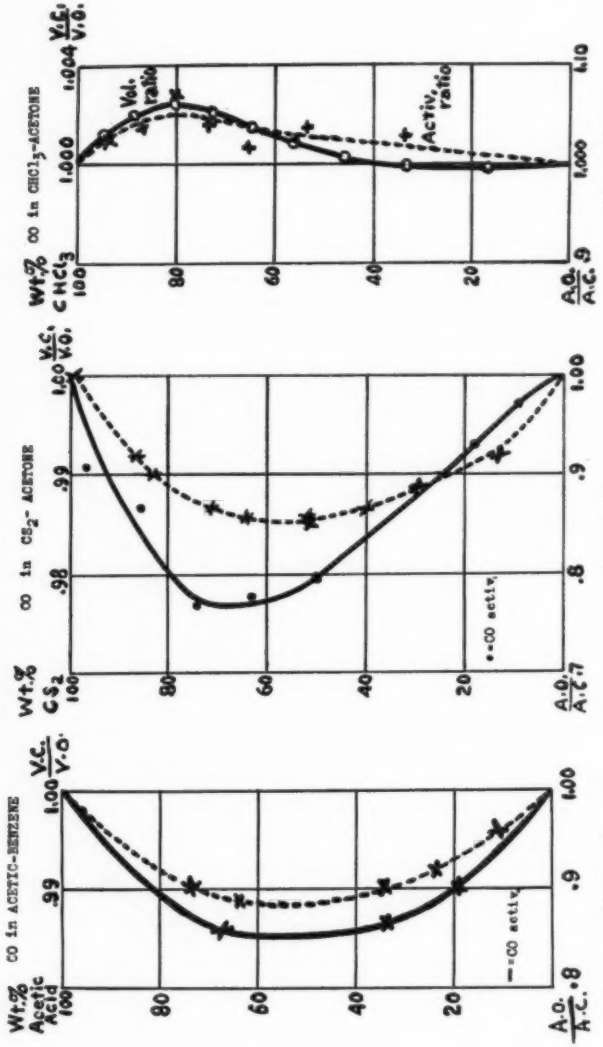

Chart V.

Chart VI.

Chart VII.

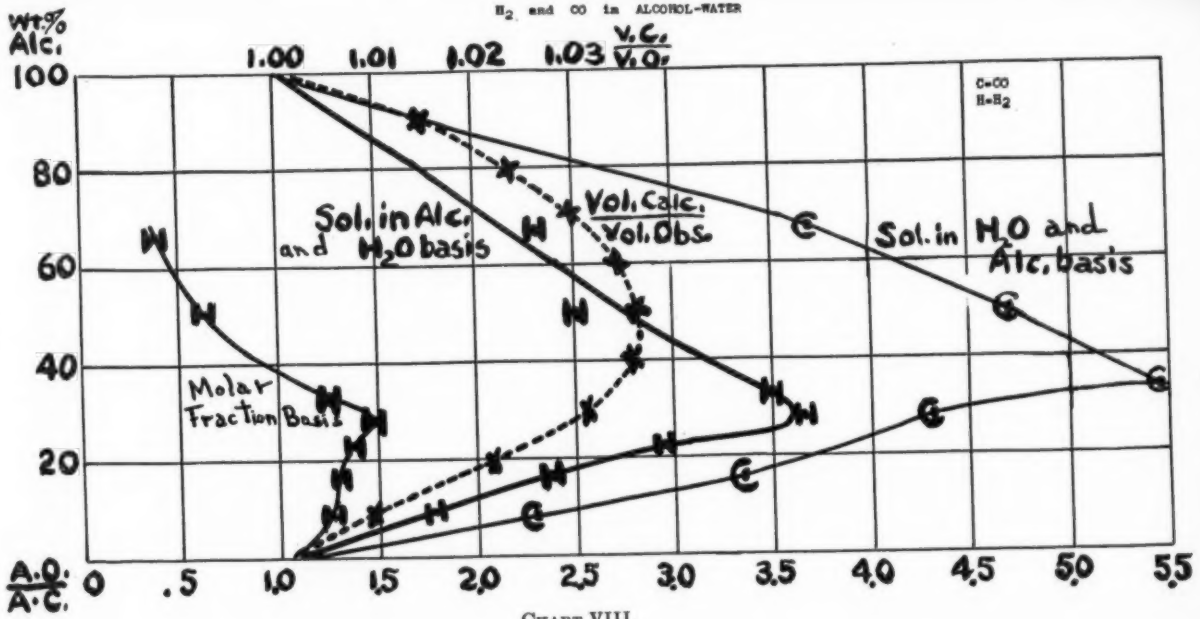
H_2 and CO in ALCOHOL-WATER
Wt.% Alc.
100
80
60
40
20
A.O./A.C.
0
.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
1.00
1.01
1.02
1.03
V.C./V.O.
C=CO
H=H_2
Sol. in Alc. and H_2O basis
Vol. Calc./Vol. Obs.
Sol. in H_2O and Alc. basis
Molar Fraction Basis

CHART VIII.

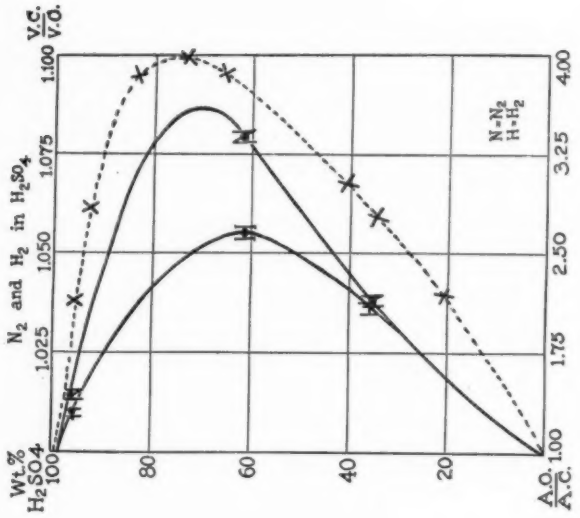

Chart X.

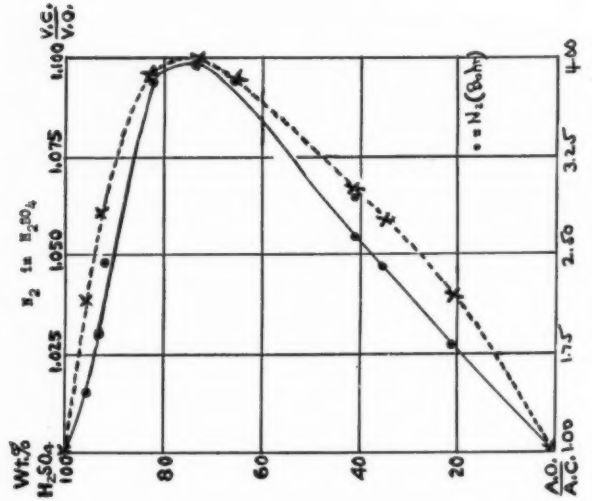

Chart IX.

CHART IX.

CHART X.

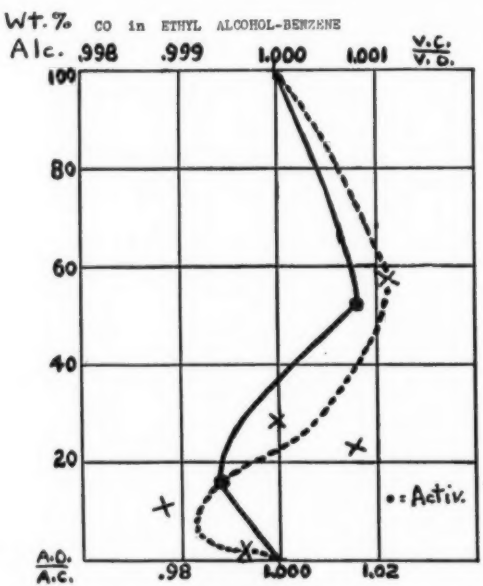

CHART XI.

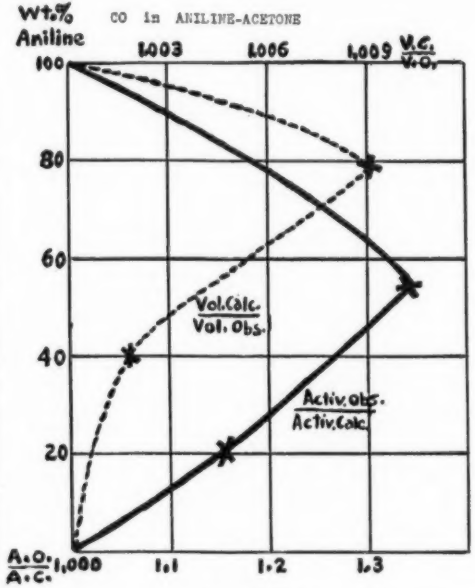

CHART XII.

CHART XIII.

CHART XIV.

CHART XV.

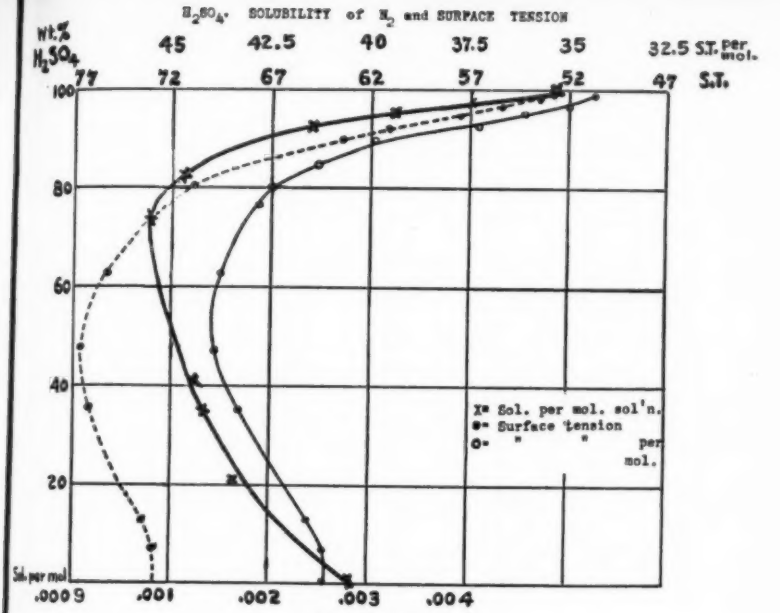

CHART XVI.

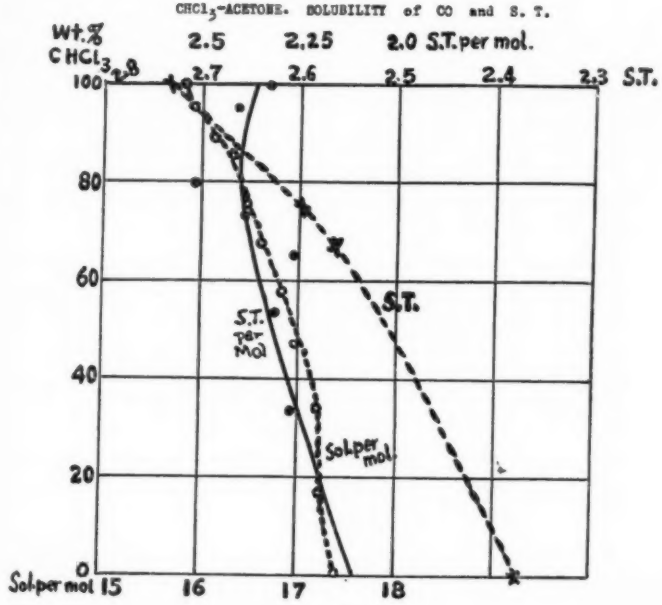

CHART XVII.

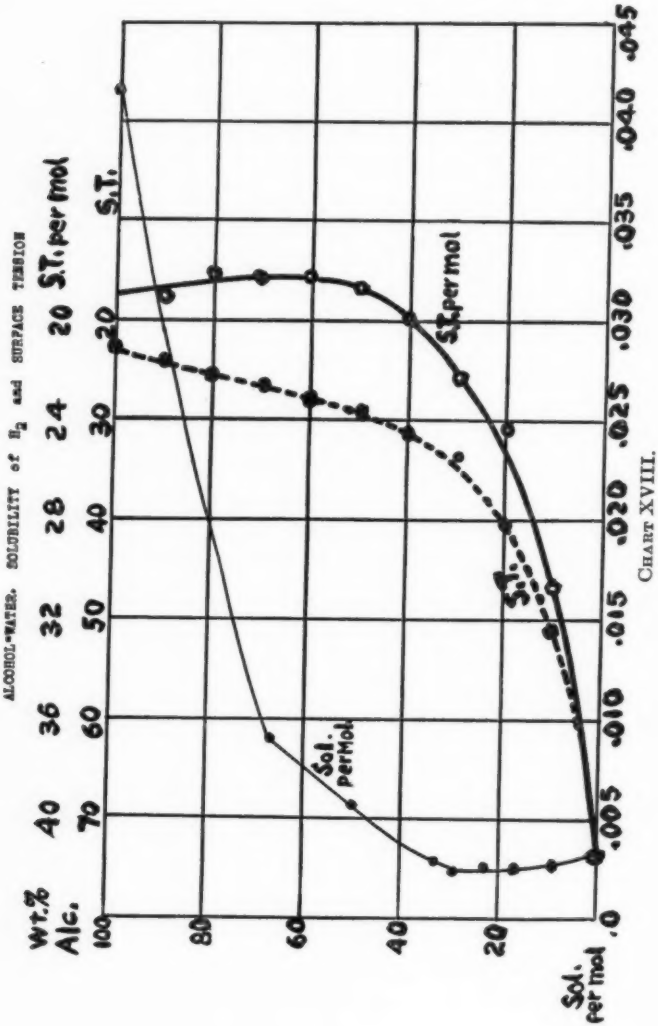
ALCOHOL-WATER. SOLUBILITY of H_2 and SURFACE TENSION
Wt.% Alc.
40 36 32 28 24 20
70 60 50 40 30 20
100 80 60 40 20 0
S.T.
S.T. per mol
Sol. per Mol
S.T.
S.T. per mol
.005 .010 .015 .020 .025 .030 .035 .040 .045
Sol. per mol

CHART XVIII.

THE PRESENCE OF AN UNKNOWN SUBSTANCE IN DIABETES INSIPIDUS URINE.

By A. B. ILLIEVITZ.

(From the Department of Biochemistry, McGill University, Montreal, Canada.)

(Received for publication, November 16, 1926.)

During the study of a case of diabetes insipidus, a substance of unknown chemical composition was isolated from the urine. The possible presence of this substance was suggested by the peculiar macroscopic changes in the urine on standing.

The urine was collected on April 15, 1924, in a flask containing a few crystals of thymol. It was kept in a refrigerator. 3 weeks later on macroscopic examination the fluid content of the whole bottle presented a purplish coloration. There was a similar coloration of the small amount of sediment. The specimen was further allowed to stand for about 2 months. The above colored substance deepened to a definite purple, and could be extracted with ether. On evaporation of the ether the material was found to be viscid. When mixed with water it was found to be insoluble and floated. On shaking it up with water, small oily globules were produced which soon rose to the surface. It is soluble in ether and chloroform, partly soluble in 75 per cent alcohol, but precipitates with absolute alcohol, forming a fine amorphous purplish sediment. Strong and weak acids do not affect it. It is slowly and totally soluble in strong alkalies, forming a colorless solution, and can be reprecipitated by means of strong acids. Hydrogen peroxide in the presence of strong ammonia causes the evolution of a gas, the precipitation of an amorphous substance, which has a faint purplish color, and the formation of an oily substance on the surface of the fluid, which has a pink coloration. An ethereal solution and a slide of the ethereal extract show no absorption bands in the spectrum.

CHART XVIII.

XUM

An attempt was made to establish its relationship to indican or tryptophane. The urine from which the unknown substance was extracted contained neither indican nor tryptophane. The urines of April 13 and 14 also showed a faint purplish sediment. All other specimens of urine failed to show the presence of indican and tryptophane in detectable quantities.

COMPARATIVE METABOLISM OF CERTAIN AROMATIC ACIDS.

XII. FATE OF TRIPHENYLACETIC ACID, ALSO TRIPHENYLMETHANE, AND TRIPHENYLCARBINOL IN THE ANIMAL BODY.

By S. R. MIRIAM, J. T. WOLF, and CARL P. SHERWIN.

(From the Chemical Research Laboratory of Fordham University, New York.)

(Received for publication, December 2, 1926.)

The study of the fate in the body of triphenylacetic acid, triphenylchlormethane, and triphenylcarbinol was undertaken for purposes of comparison with phenylacetic acid and diphenylacetic acid, which have already been investigated in this laboratory.

Triphenylacetic Acid.

Triphenylacetic acid was prepared from triphenylchlormethane by Grignard's reaction according to the method of Schmidlin,[1] as it promised relatively the largest yield. Fresh powdered magnesium was employed instead of the ribbon. By the use of 50 per cent sulfuric acid and thorough cooling during acidification, the colloidal condition of the triphenylacetic acid was avoided. 75 per cent of the theoretical amount was easily obtained. The glycocoll compound was synthesized to serve for identification of a possible metabolic product.

Triphenylacetyl Chloride.

10 gm. of triphenylacetic acid were heated with 50 gm. of thionyl chloride on an oil bath under a reflux condenser for 2 hours. The excess thionyl chloride was distilled off and the acid chloride was extracted with absolute ether. After recrystallization from benzene, 6 gm. of a yellowish white solid melting at 115° were obtained.

[1] Schmidlin, J., and Hodgson, H., *Ber. chem. Ges.*, 1908, lxi, 438.

Triphenylacetyl Glycocoll.

1.5 gm. of glycocoll were dissolved in a small flask with a minimum amount of hot water and 6 gm. of triphenylacetyl chloride were added gradually in the course of 3 hours. 10 per cent sodium hydroxide solution was used to keep the mixture alkaline; vigorous shaking and boiling were continued during the entire period. The contents of the flask were filtered hot before acidification. By fractional crystallization from chloroform and hot water, triphenylacetyl glycocoll was separated. Final purification was made by dissolving in methyl alcohol and again washing with hot water. 3.1 gm. of the substance were obtained in the form of rhombic crystals. Dried *in vacuo* at 40°, it melts at 202–204°. It is almost insoluble in water, but soluble in ether, chloroform, and methyl alcohol.

Kjeldahl Analysis for Nitrogen.

0.1480 gm. substance required 4.2 cc. 0.1 N acid; 3.97 per cent N.
0.1519 " " " 4.43 " 0.1 " " 4.08 " " "
Calculated for $C_{22}H_{19}O_3N$. N 4.06.

Micro Analysis for Hydrogen and Carbon.

10.12 mg. substance gave 5.013 mg. H_2O; 5.50 per cent H.
10.10 " " " 4.992 " " 5.49 " " "
6.60 " " " 18.546 " CO_2; 76.64 " " C.
5.012 " " " 14.104 " " 76.74 " " "
Calculated. H 5.51, C. 76.52.

Triphenylacetic acid was fed by stomach tube in the form of colloidal suspension of the sodium salt to young rabbits in ½ gm. doses on alternate days. The dose was later increased to 1 gm. Total, urea, and ammonia nitrogen, and inorganic, ethereal, and neutral sulfur were determined daily. The urine, collected for 48 hours after feeding 1 gm. of triphenylacetic acid to each of two rabbits on 2 consecutive days, was neutralized, evaporated, acidified to Congo red, and extracted in a rotary extractor successively with ether, chloroform, and ethyl acetate. Only 1.2 gm., 30 per cent, of the free acid were recovered. Examination of the feces yielded an ether extract from which 0.25 gm. of triphenylmethane, m.p. 92°, was obtained.

After feeding 1 gm. of triphenylacetic acid to a rabbit, the urine

was collected for 48 hours and acidified to Congo red. The white precipitate was extracted with ether, dissolved in 10 per cent sodium hydroxide, the solution was filtered, and the filtrate was reprecipitated with 50 per cent sulfuric acid. 0.65 gm. (65 per cent) of triphenylacetic acid was recovered.

A dog of 20 kilos, kept on a bread and milk diet during feeding and collection, was fed 2 gm. of triphenylacetic acid in one dose, as solution of the sodium salt by means of stomach tube. The dog was nervous and without appetite for 3 or 4 hours. The urine for 48 hours after the feeding was acidified with 2 per cent of its volume of concentrated sulfuric acid and extracted twice for 2 hour periods with 500 cc. of ether in a commercial electric shaker. 1.12 gm. of triphenylacetic acid were recovered.

Triphenylmethane.

On account of its insolubility in water, this substance was fed as a suspension in evaporated milk or when fed to rabbits was simply mixed with bran.

Doses of ½ and 1 gm. produced no toxic effect on rabbits of 7 months and later healthy rabbits of 11 months were able to take 4 gm. per day quite comfortably. All urine tests were negative, except for a faint Millon. 1 gm. of triphenylmethane was fed to each of two rabbits on 2 consecutive days. The urine was collected for 48 hours after the last feeding and extracted in the usual way for ten 3 hour periods with 95 per cent ethyl acetate. After repeated crystallization from hot alcohol, 1.1 gm. of triphenylmethane, 27.5 per cent of the amount fed, were recovered. Examination of the feces failed to indicate more than traces of the unchanged substance.

6 gm. of triphenylmethane were fed to a dog in 3 gm. doses on 2 successive days. The urine collection gave a faint reduction with Benedict's reagent. When treated in the manner described above, it yielded 2.1 gm. of triphenylmethane, or 35 per cent of the amount fed. The feces were not examined.

Triphenylcarbinol.

Rabbits were fed doses of 2 gm. of triphenylcarbinol with no noticeable effects. Nitrogen and sulfur determinations made on the urine showed little deviation from normal.

A rabbit was fed 4 gm. of triphenylcarbinol in 2 gm. doses on successive days. The urine was collected for 60 hours after the last feeding and extracted in the usual manner. Only the merest trace of triphenylcarbinol was found. The ether extract of rabbit urine after a feeding of a 2 gm. dose of the substance gave a yield of approximately 0.25 gm. of the unchanged substance. Apparently most of the material on account of its insolubility went through unchanged into the feces.

After feeding 5 gm. to a rabbit the feces were collected for a period of 7 days then repeatedly extracted with ether. In the ether extract 1.5 gm. of the unchanged substance were found.

XUM

COD LIVER OIL CONCENTRATE.

ITS VALUE AS AN ANTIRACHITIC AGENT WHEN INJECTED SUBCUTANEOUSLY.*

By BENJAMIN KRAMER, SIDNEY D. KRAMER, DAVID H. SHELLING,† and M. J. SHEAR.

(From the Harry Caplin Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, New York.)

(Received for publication, December 2, 1926.)

Attempts to cure rickets by subcutaneous injection of cod liver oil or its concentrate were unsuccessful in the hands of Zucker and Matzner (1) and Lesné and Vaglianos (2). The former used a cod liver oil concentrate dissolved in olive oil and the latter employed cod liver oil itself. The failure to cure rickets by this means led them to conclude that the antirachitic factor acts exclusively through the gastrointestinal tract. Soames (3) obtained moderate healing of rickets within 21 days when cod liver oil was injected into rachitic rats, either subcutaneously or intraperitoneally. At autopsy most of the oil was found unabsorbed.

Hess, Weinstock, and Hellman (4), however, were successful in curing experimental rickets with aqueous suspensions of irradiated cholesterol injected subcutaneously. Wilkins and Kramer (5) produced healing in the bones of two children suffering with active rickets by injecting a concentrate of cod liver oil dissolved in ether.

The latter findings suggested that the failures attributed to the subcutaneous route do not depend so much upon the inactivity of the injected substance as upon the solvent used. The following observations, although primarily intended as a study of the potency of cod liver oil concentrate, shed some light on the dis-

* A preliminary report of this investigation appeared in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiv, 51.

† Harry Caplin Research Fellow.

crepancies just cited. The experimental results indicate that a cod liver oil concentrate dissolved in ether and injected subcutaneously cures experimental rickets in rats. The same material, when injected with palmitin as the solvent, may yield entirely negative results.

EXPERIMENTAL.

The material used was a non-saponifiable concentrate of cod liver oil prepared according to the method of Dubin and Funk (6), and kindly supplied to us through the courtesy of Dr. Harry E. Dubin of the H. A. Metz Research Laboratories. Before any fractionation was attempted the concentrate was tested and found to be biologically potent, curing experimental rickets within 14 days in amounts equivalent to 2 cc. of cod liver oil per rat for the entire period (Table I). It also gave a strongly positive reaction with the aniline-hydrochloric acid reagent of Shear (7).

The further concentration of the non-saponifiable fraction of cod liver oil was carried out according to Drummond, Channon, and Coward (8), except that the entire procedure was executed in the open. A weighed amount of the crude fraction was extracted with twice its weight of hot methyl alcohol and allowed to cool. It was then filtered and washed several times with cold methyl alcohol until the cholesterol precipitate was only slightly yellow. The washings were added to the first filtrate and the whole evaporated on a steam bath until all the alcohol was driven off, leaving a yellow oily residue. This was marked Concentrate A.

A weighed portion of this oily substance was taken up in ethyl alcohol to make a 1 per cent solution, and the cholesterol removed with a 1 per cent solution of digitalin. The excess digitonin was removed by the method of Lifschütz (9), which consists essentially, in first diluting this alcoholic filtrate with a large amount of water and then shaking out with ether. The ether solution was subsequently washed 3 times with water. The ether-alcohol layer obtained from this last step was run into a weighed beaker, the solvents evaporated off, and the beaker again weighed. The brownish yellow oily residue obtained was marked Concentrate B.

Several samples of the crude non-saponifiable fraction were treated as outlined above and the yields of the fractions obtained were calculated and found to be approximately alike. A single example will serve to illustrate: 8.685 gm. of the crude concen-

TABLE I.

Date of autopsy.	Rat No.	Normal period.	Steenbock diet.	Test period.	Test material.	Weight. Experimental.	Weight. Final.	Histological findings.
1926		*days*	*days*	*days*		*gm.*	*gm.*	
July 23	213	25	28	14	0.02 gm. crude non-saponifiable concentrate = 2 cc. cod liver oil per rat incorporated in diet with 3 per cent olive oil.	49	51	Advanced healing.
	214	25	28	14	" "	46	54	Almost complete healing.
	215	25	28	14	" "	57	62	Advanced healing.
	216	25	28	14	" "	49	54	" "
	217	25	28	14	" "	49	54	" "
July 23	218	22	26	14	Ether solution of 12.2 mg. Concentrate A, 2 injections 7 days apart = 4 cc. cod liver oil per rat.	50	56	Advanced healing.
	219	22	28	14	" "	46	52	" "
	220	22	26	14	" "	69	73	" "
	222	22	26	14	" "	59	64	" "
	223	22	26	14	" "	72	76	" "
July 23	258A	25	28	14	Control, Steenbock diet alone.	28*	50	No healing.
	259A	25	28	14	" " " "	32*	59	" "
	260A	25	28	14	" " " "	25*	48	" "
	261A	25	28	14	" " " "	25*	39	" "

* Initial weight.

TABLE II.

Date of autopsy.	Rat No.	Normal period.	Steenbock diet.	Test period.	Test material.	Weight. Initial.	Weight. Experimental.	Weight. Final.	Histological findings.
1926		*days*	*days*	*days*		*gm.*	*gm.*	*gm.*	
Sept. 12	282	35	23	14	5.15 mg. Concentrate B incorporated in diet with 3 per cent cottonseed oil = 2 cc. cod liver oil per rat.	43	59	52	Beginning healing; calcification beginning at provisional zone covering one-half metaphysis.
	283	35	23	14	" "	41	55	57	Moderate healing.
	284	35	23	14	" "	35	43	42	" "
	285	35	23	14	" "	34	52	53	" "
	286	35	23	14	" "	46	66	69	Advanced healing.
	287	35	23	14	" "	43	65	65	Moderate "
Sept. 11	288A	37	23	13	Steenbock diet plus 3 per cent cottonseed oil.	48		77	No healing.
	289A	37	23	13	" "	42		60	" "
	290A	37	23	13	" "	45		66	" "
	293A	37	23	13	" "	40		52	" "
	294A	37	23	13	" "	37		54	" "
	295A	37	23	13	" "	41		65	" "
	296A	37	23	13	" "	37		65	" "

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Sept. 12	265	34	23	14	Single injection with ether solution of 5.15 mg. Concentrate B = 2 cc. cod liver oil.	61	90	98	Advanced healing.
	266	34	23	14	" "	63	82	82	" "
	269A	34	23	14	" "	60	73	77	" "
Sept. 11	267	34	23	13	" "	63	88	92	" "
	269	34	23	13	" "	71	100	108	Almost complete healing.
Sept. 12	264	34	23	14	Injected with ether alone.	60	73	77	No healing.
	268	34	23	14	" " " "	65	88	93	" "
	276	35	23	14	Injected with 5.15 mg. Concentrate B in palmitin = 2 cc. cod liver oil.	51	65	68	" "
	277	35	23	14	" "	38	55	60	" "
	278	35	23	14	" "	36	49	57	" "
	279	35	23	14	" "	38	46	52	" "
	280	35	23	14	" "	52	69	75	" "
	281	35	23	14	" "	45	59	62	Very slight calcification.
Sept. 12	270	32	23	14	Single injection of palmitin alone (control).	46	64	73	No healing.
	271	32	23	14	" "	45	67	72	" "
	272	32	23	14	" "	42	59	66	" "
Sept. 11	275A	32	23	13	Steenbock diet only (control).	34		64	No healing.
	301A	32	23	13	" " " "	35		65	" "
	303A	32	23	13	" " " "	33		61	" "
	305A	32	23	13	" " " "	38		74	" "
Sept. 12	273A	32	23	14	" " " "	35		60	" "
	300A	32	23	14	" " " "	46		67	" "
	302A	32	23	14	" " " "	35		66	" "

trate used yielded 2.889 gm. of Fraction A and 2.234 gm. of Fraction B; that is, about 25 per cent of the non-saponifiable material is not precipitated by digitonin. Nelson and Steenbock (10), using petroleum ether and removing the excess of digitonin by cholesterol, obtained a 50 per cent yield from the non-saponifiable fraction of cod liver oil. However, the method they employed in removing the excess digitonin differed from ours in that it necessitated the addition of cholesterol. Although our Fraction B still gave a Liebermann-Burchard reaction, it does not follow that it must have contained cholesterol, since derivatives of cholesterol react toward this reagent more strongly than cholesterol itself in a slightly modified form.

The fractions obtained were dissolved in a definite amount of ether and the cod liver oil equivalent calculated. Both Concentrates A and B gave a positive reaction with aniline-hydrochloric acid. For the biological experiments the test materials protected by an oil were incorporated in the diet or injected subcutaneously using ether or palmitin as a vehicle.

Some difficulty was experienced at first in the use of ether as a vehicle for injection. It was found that if more than $\frac{1}{8}$ cc. of ether was used for a rat weighing about 40 gm. the animal either died or became sick. In later experiments, care was exercised to concentrate the material so that the amount of ether injected did not exceed $\frac{1}{8}$ cc., and wherever possible larger rats were chosen for the tests.

Rats from our own stock were put on the Steenbock Diet 2965 when they were 22 to 37 days of age. They were kept on this diet until a severe rickets was produced, at which time they were weighed, injected with the test material, or fed on the rickets-producing diet into which the test material had been incorporated. At the expiration of the test period the animals were weighed and autopsied. Sections of the upper part of the tibiæ were immersed in 1 per cent $AgNO_3$ solution and observed under the binocular microscope for presence or absence of calcification in the provisional zone of cartilage and metaphysis.

Results.

The experimental results are tabulated in the accompanying tables. From Table I it is seen that the non-saponifiable fraction

of cod liver oil incorporated in oil when added to a rickets-producing diet cured experimental rickets in rats when fed in amounts equivalent to 2 cc. of cod liver oil per rat for the entire test period. Concentrate A or the non-saponifiable fraction from which the bulk of cholesterol has been removed by methyl alcohol is equally potent when injected subcutaneously in ether solution. Table II indicates that the cholesterol-free fraction of cod liver oil or Concentrate B still possesses antirachitic properties when administered orally or injected subcutaneously in ether solution. However, when the same concentrate was injected subcutaneously with palmitin as the solvent only one out of six rats showed the slightest amount of calcification in one or two tongues of cartilage, while the remaining five did not exhibit the slightest evidence of healing. The solvents used were found to be inactive when injected or given orally.

DISCUSSION.

After summarizing the various hypotheses that have been advanced to explain the action of cod liver oil and the experimental data in support of these theories, Schloss (11) concludes as follows: "Sicher nachgewiesen ist bisher jedenfalls nur die Darmwirkung und zur Not sind wir imstande, damit allein den therapeutischen Erfolg in der Hauptsache zu erklären."

This conclusion is supported by the fact that the administration of cod liver oil to rachitic children produces a marked diminution in the excretion of calcium and phosphate in the feces. The work of Zucker and Matzner and that of Lesné and Vaglianos referred to above, in which cod liver oil was shown to be ineffective when administered parenterally, also support this contention. However, the very careful studies of Orr, Holt, Wilkins, and Boone (12) clearly prove that an agent which acts parenterally (ultraviolet irradiation) may affect the excretion of calcium and phosphorus in exactly the same way as cod liver oil does when given by mouth. The observations of Hess, Weinstock, and Hellman, as well as those of Wilkins and Kramer and our own indicate that the antirachitic factor when injected subcutaneously produces healing of the rachitic process. This consists not only in a deposition of bone-forming salts but if the treatment is sufficiently prolonged, there is a disappearance of the metaphysis and

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the tongues of cartilage so that eventually the normal osseous architecture is reestablished. This speaks for an effect other than that exerted in the gastrointestinal tract. It might be argued that the active principle may find its way from the point of injection into the intestine. However, there is no evidence in support of such a contention.

CONCLUSIONS.

1. A cholesterol-free concentrate prepared by the method described was found to be as potent as cod liver oil itself in amounts equivalent to the original oil.

2. The concentrate when injected subcutaneously in an ether solution cured experimental rickets in rats.

3. The same concentrate is inactive when injected subcutaneously with palmitin as the solvent.

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CHANGES IN THE SUGAR, INORGANIC PHOSPHORUS, AND LACTIC ACID OF ANIMAL AND HUMAN BLOOD AFTER ADMINISTRATION OF INSULIN AND OF GLUCOSE.

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INTRODUCTION.

In a recent paper Briggs, Koechig, Doisy, and Weber (1) have reported a decrease in the concentration of glucose, inorganic phosphorus, and potassium in the blood of normal animals after insulin. There was observed a parallel increase in the lactic acid from 100 to 400 per cent of the control figure, apparently formed from glucose under the influence of insulin. The animals were not anesthetized and some developed convulsions, but the authors believe that the increased muscular activity played no part in the observed production of lactic acid. On the other hand Best and Ridout (2) state that the lactic acid of dogs does not significantly increase during insulin hypoglycemia except when this condition is associated with extreme asthenia or increased hyperirritability of the neuromuscular system. Cori (3) has also found that insulin hypoglycemia produced no definite changes in the lactic acid content of the blood of either fasting rabbits or cats. Moreover the blood lactic acid of phlorhizinized rabbits and depancreatized cats was not influenced by the insulin, although the blood lactic acid was markedly increased by insulin convulsions. Tolstoi, Loebel, Levine, and Richardson (4) conclude from their observations on the bloods of diabetics that insulin does not act by converting glucose into lactic acid. Although the insulin caused a rise in the blood lactic acid, the effect was far from constant. There was observed no quantitative relation between the blood sugar decrease and the lactic acid produced. Moreover adrenalin produced a hyperglycemia accompanied by an increase in the lactic acid. These authors believe the rise in lactic acid might be due to a local asphyxia. Both insulin and adrenalin lowered the inorganic phosphorus of the blood.

Wigglesworth, Woodrow, Smith, and Winter (5) report a rapid fall in the inorganic phosphorus of the blood of rabbits following the injection of insulin, and this low level was maintained for many hours after the relief

of the insulin convulsions by glucose. The total acid-soluble phosphorus was very little altered. Striking variations in the excretion of phosphate following the ingestion of sugar or insulin administration to dogs have been noted by Sokhey and Allan (6). Changes in the inorganic phosphorus of the blood and urine of normal and diabetic subjects after ingestion of glucose accompanied by the intravenous administration of insulin have been studied by Perlzweig, Latham, and Keefer (7). A marked decrease in the inorganic phosphorus of the blood was found and a simultaneous fall in the rate of excretion of phosphate in the urine. Similar observations have been reported by Blatherwick, Bell, and Hill (8) who have noted that insulin administered to normal individuals before glucose ingestion causes a pronounced decrease in the inorganic phosphorus of the blood. At the same time the rate of excretion of phosphorus in the urine is diminished. These changes were seen during the period of the hypoglycemia.

The results of their observations upon human subjects and rabbits have led Harrop and Benedict (9) to the conclusion that during active carbohydrate assimilation there was a shift in the mobile phosphate stores of the body. This shift was indicated by a lessened phosphate excretion in the urine, a decreased concentration in the blood during this period, and a phosphate retention in the muscle tissue. The phosphate stored in the muscle tissue was later released. The shift of phosphates following insulin injection, these authors found to be a constant phenomenon. Kay and Robison (10) have shown that after insulin administration phosphoric esters are synthesized in the corpuscles at the expense of inorganic phosphate originally present in the blood plus additional phosphate drawn from other tissues. The experiments of Audova and Wagner (11) also demonstrate a retention of phosphate in the muscles after insulin. However, Sokhey and Allan state that the study of the lactacidogen and inorganic phosphate content of rabbit muscle after insulin and insulin with sugar has not confirmed the conclusions of Harrop and Benedict, nor those of Audova and Wagner.

The recently reported experiments of Cori and Cori (12) are of interest in connection with Harrop and Benedict's observations on the phosphate retention in the muscle tissue after insulin. Cori and Cori noted that insulinized rats stored less glycogen and oxidized more glucose than the controls receiving similar amounts of glucose. In the insulinized rats there was less liver glycogen deposited, and this difference in liver glycogen accounted for the difference in the total glycogen of the two groups of rats. But the insulinized animals deposited in the rest of the body tissues, chiefly the muscles, the same amount or even more glycogen than the normal animals.

Elias and Weiss (13) have demonstrated a definite effect upon carbohydrate metabolism of phosphate administrations. The intravenous injection of hypertonic solutions of NaH_2PO_4 and Na_2HPO_4 produced a decrease in the blood sugar in diabetic and alimentary hyperglycemias, while the normal blood sugar remained unchanged. The blood sugar fall was accompanied by a diminished sugar excretion; the fall in blood sugar per-

sisted for 2 to 4 hours, and the urine sugar sometimes for 3 days. Kolodziejska and Funk (14) have studied the influence of insulin upon the total phosphorus metabolism of rats. They note that the total phosphorus metabolism remains unchanged. The bodies of the insulinized rats when analyzed *in toto* showed no variations in their phosphorus content from that of the controls maintained on the same diet. The changes in the phosphorus of the blood and urine observed by all workers after insulin administrations were confined to very short intervals, and such changes would be obscured in specimens of urine or feces collected over long intervals.

The evidence presented in the literature appears to lend further proof to the hypothesis that an intermediate phosphate compound with carbohydrate is formed in the utilization of glucose subsequent to glucose or insulin administration. However, there is an apparent contradiction in the reported data on the changes in the blood lactic acid associated with insulin hypoglycemia. The object of the experiments reported here was to determine the relation of the changes in lactic acid to variations in the inorganic phosphorus and sugar of the blood in rabbits and human subjects, normal and diabetic. It is significant, we believe, that the literature contains no observations on the influence of insulin on the lactic acid of man except for the incomplete data of Tolstoi, Loebel, Levine, and Richardson.

EXPERIMENTAL.

As a preliminary to the observations on human subjects it was deemed wise to repeat some of the experiments on animals. For this purpose twelve rabbits were used, averaging in body weight 2 kilos. Of these, seven were anesthetized with ethyl urethane; enough of the urethane in a 50 per cent solution was given in a subcutaneous injection (2 cc.) to produce muscular relaxation without a deep coma. When the rabbits showed definite evidence of the anesthesia (about 90 minutes), six received the insulin,[1] one did not. The remaining animals (five) were not anesthetized. In all cases the control bloods were obtained after a 24 hour fast, and during the experiments all food was withheld. The blood was drawn from the ear veins and analyzed immediately. The blood sugar was determined by the method of Folin and Wu, and the

[1] The insulin used was that manufactured and placed on the market by Eli Lilly and Company, under the trade name of iletin.

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TABLE I.

Rabbit No.	Time.	Blood.		Remarks.
		Sugar.	Lactic acid.	
		mg. per 100 cc.	*mg. per 100 cc.*	
1	12.10 p.m.	124	12.6	12.15 p.m. 2 cc. of 50 per cent urethane injected.
	1.15 "	125	13.1	
	2.15 "	121	17.6	
	4.15 "	115	13.6	
2	1.40 p.m.	115	16.4	10.00 a.m. 2 cc. of 50 per cent urethane injected. 1.40 p.m. 2.5 units insulin per kilo injected. No convulsions.
	3.40 "	75	17.0	
	5.40 "	66	17.0	
	7.00 "	55	20.1	
3	11.30 a.m.	121	16.3	10.00 a.m. 2 cc. of 50 per cent urethane injected. 11.30 a.m. 2.5 units insulin per kilo injected. No convulsions.
	1.30 p.m.	64	16.6	
	3.30 "	76		
	5.30 "	96	16.4	
4	12.00 m.	109	26.5	Control. During urethane anesthesia 12.15 p.m. 10 units insulin per kilo injected. No convulsions.
	1.25 p.m.	101	31.9	
	2.35 "	54	23.8	
	3.30 "	34	29.7	
5	12.15 p.m.	130	18.9	Control. During urethane anesthesia 12.20 p.m. 5 units insulin per kilo injected. No convulsions.
	1.38 "	38	20.2	
	2.15 "	35	26.1	
6	11.55 a.m.	125	24.7	Control. During urethane anesthesia 12.00 m. 2.5 units insulin per kilo injected. No convulvulsions.
	1.20 p.m.	65	19.3	
	2.20 "	53	25.7	
	3.20 "	47	28.3	
	4.20 "	50	21.2	
7	11.00 a.m.	130	20.3	Control. During urethane anesthesia 11.00 a.m. 1 unit insulin per kilo injected. No convulsions.
	12.00 m.	59	18.5	
	1.05 p.m.	35	22.0	
	2.03 "	40	21.2	
8	10.20 a.m.	126	18.9	10.30 a.m. 2.5 units insulin per kilo injected. 2.00 p.m. convulsions (mild).
	12.30 p.m.	68	20.1	
	2.30 "	35	19.4	
	4.30 "	31	19.4	
9	11.00 a.m.	125	15.1	11.20 a.m. 2.5 units insulin per kilo injected. 1.30 p.m. convulsions (mild). Died.
	1.20 p.m.	51	14.5	
	3.20 "	113	25.8	

TABLE I—*Concluded.*

Rabbit No.	Time.	Blood.		Remarks.
		Sugar.	Lactic acid.	
		mg. per 100 cc.	*mg. per 100 cc.*	
10	11.00 a.m.	120	15.6	11.20 a.m. 2.5 units insulin per kilo injected. 1.20 p.m. convulsions (mild). Died.
	1.20 p.m.	30	17.3	
	3.20 "	120	20.8	
11	10.20 a.m.	113	28.3	10.30 a.m. 2.5 units insulin per kilo injected. No convulsions.
	12.30 p.m.	35	25.8	
	2.30 "	113	43.4	
12	10.20 a.m.	105	12.3	10.30 a.m. 2.5 units insulin per kilo injected. No convulsions.
	12.30 p.m.	48	30.2	
	2.30 "	47	28.6	
	4.30 "	55	28.3	

procedure described by Clausen was utilized for the lactic acid estimations, with potassium permanganate as the oxidizing agent. During the 4 hours of urethane anesthesia in Rabbit 1 (Table I) no significant change is noted either in the blood sugar or the lactic acid. The amount of insulin administered to the animals varied from massive doses of 10 units to 1 unit per kilo of body weight. In all instances a hypoglycemia followed the insulin administration, and in four cases (Rabbits 3, 9, 10, 11) there was a subsequent return of the blood sugar to normal. No convulsions developed in the anesthetized animals during the period of observation for which the results are reported in Table I. Of the unanesthetized rabbits, three showed convulsions at the time of the hypoglycemia, and two did not. Considerable variation is evident in the lactic acid content of the control bloods, ranging from 12.3 to 26.5 mg. per 100 cc. Following the insulin administration all rabbits showed some increase in the blood lactic acid; however, the increases are not uniform. The maximum concentration of lactic acid does not correspond in time with the maximum decrease in blood sugar. Moreover there appears to be no quantitative relation between the rise in the blood lactic acid and the drop in blood sugar. However, such a relation is not to be expected if the hypothesis is correct that lactic acid is an inter-

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mediate product in carbohydrate metabolism. It is evident that from these data no conclusion can be deduced.

The influence of insulin upon the lactic acid, inorganic phosphorus, and the sugar of the blood has been studied in three normal individuals. The data are presented in Table II. A 16 hour fast preceded these observations. No food was consumed during the experiments but water drinking was permitted. Definite hypoglycemias were produced in all three cases, but the hypoglycemia was free from all clinical symptoms. The subjects remained at rest in bed throughout the experiments. In case S. B. the blood sugar dropped from 104 to 51 mg. per 100 cc. in 3 hours, and then

TABLE II.

Influence of Insulin upon Sugar, Lactic Acid, and Inorganic Phosphorus of Blood of Normal Adults during Fasting State.

	Subject A. C.		Subject S. B.			Subject J. B.		
	Sugar.	Lactic acid.	Sugar.	Lactic acid.	Inorganic P.	Sugar.	Lactic acid.	Inorganic P.
Control	84	10.1	104	13.8	4.3	93	10.1	4.2
Insulin, 10 units.								
1st hr	105	8.0	93	20.9	4.0	84	18.6	4.3
2nd "	73	26.8	71	16.4	4.0	78	23.7	4.0
3rd "	76	34.7	51	17.5	3.9	72	31.7	4.4
4th "	81	27.2	60	19.4	4.5	66	23.9	4.3
5th "	69	39.2	71	9.8	4.3	68	19.9	4.5

Figures are for mg. per 100 cc. of blood.

returned towards normal. In the other two cases the decrease in blood sugar was much less rapid.

Clausen (15) at the time of the description of his technique established as the provisional range of figures for the lactic acid of normal human blood 15 to 32 mg. per 100 cc. Nishimura (16) has emphasized some of the possible sources of error in the method, and has reported the lactic acid of normal human blood varying from 11 to 18, with an average of 15 mg. per 100 cc. In these experiments the subjects were maintained completely at rest for an hour before the control blood was drawn and throughout the entire period of the experiment. In drawing the blood stasis was prevented by avoiding the use of a tourniquet.

In all instances an increase in the blood lactic acid followed the administration of 10 units of insulin. The maximum figure obtained was 2 to 3 times the control concentration. In two subjects a decrease in the inorganic phosphorus of the blood serum was noted with a subsequent rise. In one instance (J. B.) the minimum inorganic phosphorus of the blood precedes in time the maximum lactic acid concentration. In the experiments of Harrop and Benedict the influence of insulin upon the inorganic phosphorus of the blood serum was much less striking than its effect upon phosphate excretion in the urine. Their data show that on the days during which insulin was given, there was a reduction in phosphate excretion throughout the hours in which

TABLE III.

Influence of Insulin after Breakfast upon Sugar and Lactic Acid of Blood of Diabetics.

	Subject P. G.		Subject P. F.		Subject I. S.	
	Sugar.	Lactic acid.	Sugar.	Lactic acid.	Sugar.	Lactic acid.
Control.....	517	14.8	185	30.1	286	12.3
Insulin, 15 units.						
1st hr.	441	19.1	250	40.0	322	17.5
2nd "	319	23.8	235	43.6	263	16.4
3rd "	273	20.2	160	49.0	231	26.5

Figures are for mg. per 100 cc. of blood.

the insulin was active. A great increase in the urinary excretion of phosphate took place during the following night when the effect of the insulin had ended.

The influence of insulin upon the lactic acid of the blood in diabetes was first studied in three subjects (Table III) who received 15 units of insulin preceding a breakfast containing 15.2 gm. of protein, 57.5 gm. of fat, and 23.3 gm. of carbohydrate. At hourly intervals thereafter for 3 hours specimens of blood were obtained. It is seen that the insulin effects a reduction in the blood sugar accompanied by a rise in the lactic acid. The rise in the lactic acid varies from 36 to 115 per cent of the control figure. In these observations, it is not possible to determine how much of the increase in the lactic acid is due to the breakfast and how much depends upon the insulin.

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An attempt was made to compare the influence of insulin on the blood lactic acid with that of glucose. Mendel, Engel, and Goldscheider (17) have observed no change in the lactic acid of the blood after the ingestion by mouth of 50 and 75 gm. of glucose in two cases. Katayama (18), however, has found that glucose taken by mouth (1.75 gm. per kilo of body weight) is followed by a rise in the lactic acid of the blood, and this rise is greater in individuals with normal tolerance for carbohydrate than in those with a diminished tolerance. The subjects of the experiments reported here were three individuals (Table IV), one of whom had a normal

TABLE IV.

Lactic Acid of Blood of Normal Subjects after Glucose and Glucose with Insulin.

	Subject R. H.		Subject A. B.		Subject S. P.	
	Sugar.	Lactic acid.	Sugar.	Lactic acid.	Sugar.	Lactic acid.
Control	98	10.6	104	10.3	113	15.1
Glucose	97 gm.		140 gm.		84 gm.	
1st hr.	134	11.7	198	14.8	221	19.3
2nd "	119	36.9	166	33.7	183	25.6
Glucose	50 gm.		50 gm.		50 gm.	
Insulin. (30 minutes later)	10 units.		10 units.		10 units.	
1st hr.	100	52.6	141	68.8	155	31.1
2nd "	90	91.3	76	100.3	125	33.3
3rd "	114	99.4	115	81.4	122	45.4

Figures are for mg. per 100 cc. of blood.

tolerance for glucose, the other two (A. B. and S. P.) a moderately diminished tolerance. All received glucose by mouth in a 50 per cent solution, 1.75 gm. per kilo of body weight. Changes in blood sugar and lactic acid were followed for 2 hours, then 50 gm. of glucose were ingested and 10 units of insulin administered. All three cases show an increase in the blood lactic acid after the glucose, but following the insulin administration the increase in lactic acid is markedly greater, 2 to 3 times that observed after the glucose alone. In subject S. P. it is seen that the glucose produces a persistent hyperglycemia, with an increase in lactic acid less than the other two cases. Also after insulin in S. P. the decrease in

TABLE V.

Lactic Acid of Blood of Diabetics after Glucose, and after Insulin.

Subject.	Hour.	Blood.		
		Sugar.	Lactic acid.	Inorganic P.
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
1. T. McA.	Control.	141	10.3	5.0
Age, 11 yrs. M.	Glucose, 56 gm.			
	1st.	238	10.1	4.2
	2nd.	344	12.1	4.0
	Insulin, 15 units.			
	1st.	275	15.1	3.0
	2nd.	144	32.5	3.6
	3rd.	59	36.8	3.8
2. V. V.	Control.	319	10.1	3.3
Age, 29 yrs. M.	Glucose, 134 gm.			
	1st.	428	9.4	3.5
	2nd.	472	19.4	3.3
	Insulin, 20 units.			
	1st.	428	26.9	3.2
	2nd.	322	26.4	2.9
	3rd.	288	46.5	3.3
3. L. G.	Control.	192	36.6	5.0
Age, 32 yrs. F.	Glucose, 103 gm.			
	1st.	282	49.7	4.8
	2nd.	326	26.4	4.4
	Insulin, 15 units.			
	1st.	306	46.8	4.3
	2nd.	262	45.9	3.2
	3rd.	214	69.5	3.2
4. J. L.	Control.	86	26.7	3.2
Age, 47 yrs. M.	Glucose, 111 gm.			
Gangrene of foot.	1st.	155	47.1	3.6
	2nd.	280	40.8	3.6
	Insulin, 20 units.			
	1st.	234	41.4	3.6
	2nd.	94	34.9	3.9
	3rd.	58	45.5	4.4
5. C. J.	Control.	248	19.7	
Age, 25 yrs. M.	Glucose, 50 gm.			
	1st.	349	28.3	
	2nd.	405	37.3	
	3rd.	390	34.6	

TABLE V—*Concluded.*

Subject.	Hour.	Blood.		
		Sugar.	Lactic acid.	Inorganic P.
		mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
5. C. J.—Continued.	Insulin, 15 units.			
Age, 25 yrs. M.	1st.	297	28.4	
	2nd.	192	35.5	
	3rd.	94	34.9	
	4th.	92	38.2	
6. A. R.	Control.	278	13.3	
Age, 58 yrs. F.	Glucose, 94 gm.			
	1st.	500	27.4	
	2nd.	667	30.2	
	Insulin, 25 units.			
	1st.	601	31.9	
	2nd.	256	36.4	
	3rd.	106	50.9	
7. J. B.	Control.	380	55.4	
Age, 9 yrs. M.	Glucose, 37 gm.			
	1st.	536	84.8	
	2nd.	759	28.2	
	Insulin, 20 units.			
	1st.	536	110.8	
	2nd.	294	62.0	
	3rd.	106	21.9	

blood sugar and the rise in lactic acid are much less than the corresponding changes in R. H. and A. B.

Similar observations have been made upon seven diabetics and these results are presented in Table V. Subjects 1 and 4 (T. McA. and J. L.) were mild, and the remainder moderately severe cases of diabetes. Subjects 3 and 4 (L. G. and J. L.) show unusually high concentrations of lactic acid in the control bloods, due, we believe, to an associated infection. The ingestion of the glucose produced a persistent hyperglycemia and a rise in the lactic acid in all instances. The increase in lactic acid, however is not as great as that observed in Table IV after glucose. In Subjects 1 and 3, the blood phosphorus was decreased, but in two others, Nos. 2 and 4, a slight increase was noted. Perlzweig, Latham, and Keefer have noted in diabetics that the glucose or

insulin did not cause a retention of phosphate, especially when the blood sugar curves and the respiratory exchange indicated that there was no increased catabolism of carbohydrate. In two of the subjects reported by Blatherwick, Bell, and Hill there is seen a slight rise in the inorganic phosphorus of the blood after glucose ingestion. Katayama has also reported an increase in the blood phosphorus after glucose in some instances.

The injection of the insulin produces a decrease in the blood sugar, accompanied by a rise in the blood lactic acid. This increase in the lactic acid, despite the larger doses of insulin, is not as great as that found in the non-diabetic cases of Table IV. With the exception of Subject 7 (J. B.), in all of the diabetics the maximum figure for the lactic acid corresponds in time with the minimum blood sugar. In J. B. the lactic acid of the control blood is about 5 times the normal figure and rises promptly after the glucose. Following the insulin we again obtained a precipitate increase in the blood lactic acid, the maximum concentration appearing at the end of the 1st hour. In the subsequent specimens of blood there is a steady decrease in both the lactic acid and the sugar. This patient at all times appeared to be in an unstable state of carbohydrate metabolism. His response to insulin therapy was always prompt and greater than that of other diabetics. 1 unit of insulin would decrease the blood sugar about 40 mg. per 100 cc. in 4 hours. His tolerance for carbohydrate was very low; however the respiratory quotient was 0.78, and the basal metabolism 2 per cent above the DuBois standard. There appears to be no quantitative relation between the drop of blood sugar and the increase in lactic acid. In Subject 4 (J. L.) the blood sugar decrease in 3 hours amounts to 222 mg. per 100 cc. but the rise in lactic acid totals only 4.7 mg., about 1 per cent of the original concentration. On the other hand, in Subject 1 (T. McA.) a fall in blood sugar of 285 mg. is accompanied by a lactic acid increase of 24.7 mg. In both of these instances the insulin produced hypoglycemias.

The inorganic phosphorus of the blood falls after the insulin in three of the diabetics, but in Subject 4 (J. L.) a slight increase is noted. We are at a loss to explain this rise in the inorganic phosphorus. In those instances in which the inorganic phosphorus

is diminished the maximum drop in the phosphorus precedes the rise in lactic acid.

All of the observations reported in the tables were made upon subjects after a night's fast of 16 hours, and during the period of the experiments no food was consumed. It is possible that the food privation may have influenced the results obtained. To answer this question, changes in the blood sugar and lactic acid of the bloods of three diabetics were followed during the last 4 hours of a 20 hour fast. The results are reported in Table VI. It is evident from these data that the fasting was without appreciable effect upon the sugar and lactic acid of the blood.

TABLE VI.

Changes in Sugar and Lactic Acid of Blood of Diabetic Adults during Fasting State.

	Subject P. G.		Subject P. F.		Subject I. S.	
	Sugar.	Lactic acid.	Sugar.	Lactic acid.	Sugar.	Lactic acid.
1st hr.	461	13.9	211	25.6	306	12.0
2nd "	500	13.0	205	22.9	322	15.7
3rd "	500	12.6	183	21.6	312	15.7
4th "	492	15.7	197	21.1	273	16.6

Figures are for mg. per 100 cc. of blood.

DISCUSSION.

From the data presented in the tables it is evident that insulin produces an increase in the lactic acid of the blood of rabbits and human subjects. In the animals the changes in concentration of lactic acid are very slight in comparison with the drop in blood sugar. Insulin, here, was given in massive doses and the drop in blood sugar is rapid. Since the increase in lactic acid appears in the anesthetized animals without convulsions, it does not seem possible that it could be due to an increased activity of the skeletal muscles. In both normal and diabetic human subjects a rise in the blood lactic acid was consistently noted after insulin administration. The rise seen in normal human blood was significantly greater than that found in the diabetics. Glucose ingestion (1.75 gm. per kilo of body weight) also elevated the blood lactic

acid, but this effect was in all instances less than that observed after insulin. Tolstoi, Loebel, Levine, and Richardson contend that the change in the lactic acid of the blood after insulin cannot be due to lactic acid produced in carbohydrate utilization since it does not bear a quantitative relation to the blood sugar loss. Such a view does not appear reasonable. In none of our experiments does the increase in lactic acid account for the drop in blood

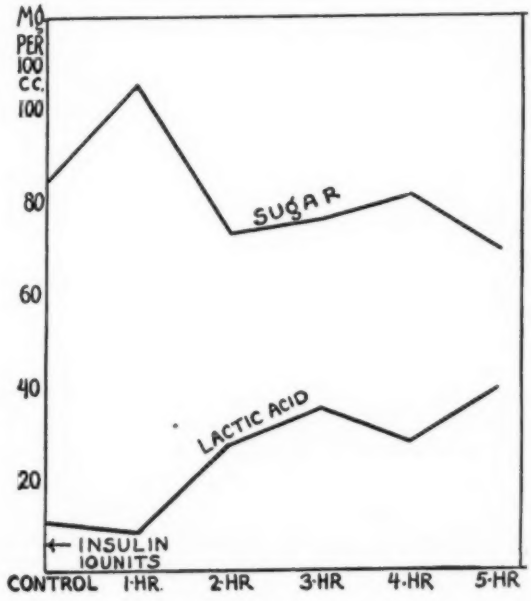

Chart I.

sugar. If the lactic acid production represents an intermediate reaction in the glucose utilization, it is not to be expected that all of the lactic acid produced would remain as such in the blood stream. With the increased rate of oxidation of the glucose produced by the insulin, the lactic acid is readily disposed of. The data presented by these authors show a distinct rise in the respiratory quotient, and an increase in the carbohydrate metabolized per hour. Moreover they have reported but one figure

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for the lactic acid after insulin, and it is possible that this figure does not represent the maximum concentration in the blood.

That there is an increased rate of oxidation of glucose after insulin administration is conclusively established by the work of Dudley and Marrian (19), Lyman, Nicholls, and McCann (20), and Ringer (21). Cori and Cori have shown that the insulinized rats oxidize more glucose than control rats. The quotient

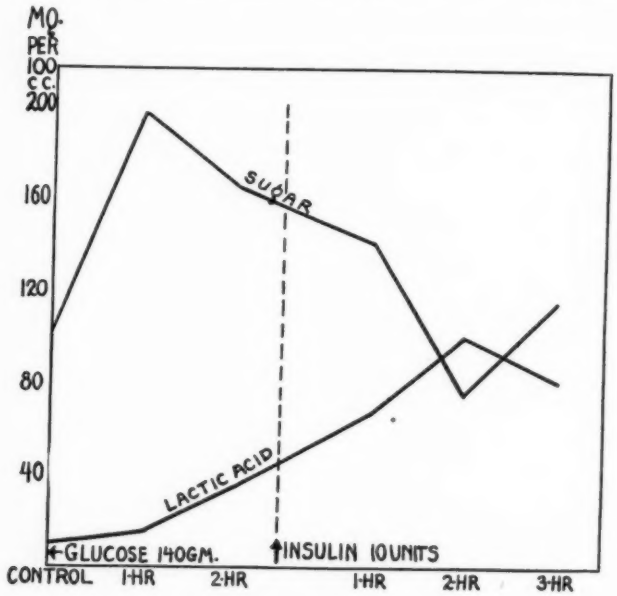

CHART II.

$\frac{\text{glycogen formed}}{\text{glucose oxidized}}$ was 1.38 for normal rats and 0.87 for insulinized rats receiving the same amount of sugar.

Morgulis and Barkus (22) have attempted to make a distinction between glycolysis *in vitro* and insulin hypoglycemia in the fact that glycolysis *in vitro* is accompanied by a production of lactic acid, but that no increase in blood lactic acid is found in insulin hypoglycemia. These authors present no figures for changes in

lactic acid of the blood after insulin; apparently their statement is based upon data in the literature. Our observations do not substantiate such distinction between glycolysis *in vitro* and insulin hypoglycemia.

Chart I presents graphically changes in the sugar and lactic acid of the blood of a normal individual (A. C., Table II) when insulin was administered after a night's fast. It is evident from the graphs that as the sugar diminishes, the lactic acid increases in concentration. The maximum figure for lactic acid and the minimum for the sugar in this experiment were obtained 5 hours after administration of 10 units of insulin. Chart II gives the curves for the sugar and lactic acid of a normal individual after glucose, followed 2 hours and 30 minutes later by insulin (A. B., Table IV). A rise in lactic acid follows the ingestion of the glucose, but this is magnified by the insulin. The increase in lactic acid here following the insulin is much greater than that of Chart I, no doubt because the previous ingestion of glucose rendered a greater supply of carbohydrate available for oxidation. Here again, the maximum figure for lactic acid corresponds in time with the greatest drop in blood sugar. All our observations are not as uniform as those presented in the charts. The data of Briggs and his associates show graphically the changes in blood sugar and lactic acid after insulin administration in dogs. Their graph represents the lactic acid reaching the maximum figure at a time when the blood sugar is lowest. They have at most but two figures for the lactic acid after insulin from 2 to 4 hours apart It is quite possible that with more frequent determinations of the blood sugar and lactic acid, their curves would not have been as smooth as represented in their Fig. 1.

The changes in the inorganic phosphorus of the blood after glucose and after insulin correspond to those reported in the literature. In our experiments it has been found that the decrease in the inorganic phosphorus of the blood precedes the rise in lactic acid.

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XUM

STUDIES OF THE SOLUBILITY OF CALCIUM SALTS.

I. THE SOLUBILITY OF CALCIUM CARBONATE IN SALT SOLUTIONS AND BIOLOGICAL FLUIDS.

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INTRODUCTION.

The biological importance of calcium has been recognized since the earliest times, but it has only been comparatively recently, through the stimulus afforded by the growing realization of the close association between some pathological conditions and disturbances of the calcium metabolism of the body, that the element calcium has claimed so much attention and study in biological fields. Being the only cation, except for small amounts of magnesium, capable of forming difficultly soluble salts with the common anions present, namely carbonate and phosphate, it carries unaided, the burden of furnishing the organism with its framework of ossified tissue, the skeleton. However, its function and purpose do not cease here. Its effect on nerve and muscle permeability and irritability alone would warrant more important consideration of the nature of its metabolism.

The work to be reported in this and subsequent papers does not represent an attempt to solve any one particular biological problem which is concerned with calcium metabolism, but is rather a quantitative investigation of certain biological systems in which calcium plays a rôle. Before work upon biological systems could be interpreted, however, much work was necessarily carried out on systems of simple composition. These will be given in the following order: First, studies of the solubility of calcium carbonate in salt solutions and biological fluids;

second, studies of the solubility of tertiary calcium phosphate in the same systems; third, studies of the solubility of both salts in similar solutions.

Studies of equilibria in heterogeneous systems composed of the solid phases, calcium carbonate and calcium phosphate, the liquid phases, aqueous solutions of various electrolytes and non-electrolytes, and the gas phase, carbon dioxide, have long attracted the attention of theoretical, technical, and biological chemists. Interest in such a system may be centered upon the liquid phase in connection with such problems as the factors influencing the activity of the ions present in it, or upon the solid phase and the factors influencing the precipitation or solution of one or more of the components. To the biologist each of these points of view is of importance.

Ionic activity, particularly that of calcium ions, is believed to be intimately related to the problem of the functional activity of nerves and muscles, and hence may be considered an important factor in tetany.

Although there is present in serum far more calcium than one could dissolve in a salt solution of the same concentration, a diminution or increase in the calcium concentration is known to result in profound biological changes. Among the most striking of these, following a decrease in its concentration, is the condition of hyperexcitability which, in the intact animal, manifests itself in the symptom syndrome known as tetany. Conditions are known, however, as for example in nephrosis, in which low serum calcium concentrations are found without symptoms of hyperexcitability. It becomes of interest therefore to inquire first into the reason for the large amount of calcium in serum and the forms in which it is present, and second into the reason for the differences in biological response following changes in calcium concentrations.

A knowledge of the factors affecting the precipitation and solution of calcium carbonate and calcium phosphate underlies the solution of such physiological problems as bone and tooth formation and such pathological problems as rickets and arteriosclerosis.

Problems concerned primarily with the nutritional aspect of calcium metabolism, involving such questions as the amount of

elementary calcium necessary for cellular activity, maintenance, and growth, are not to be considered in this work.

Another point of view has for its purpose the understanding of the behavior of calcium in the organism. Before its functional activity can be understood, however, certain knowledge concerning the physicochemical system involved, is necessary. Such questions as the following occur to one taking this point of view.

What is the biological system of which calcium is an important constituent? Is this system in equilibrium, and if so, in what kind of an equilibrium? In what forms is calcium present in this system? What are the quantitative relationships between these forms? What factors influence these relationships? Our work has had for its purpose an attempt to answer some of these and similar questions. Although final answers have not been found in all cases, in certain of them a reasonable degree of success has been met, while in others evidence which may contribute to the final solution has been obtained.

Theoretical Considerations.

Much work has been done in recent years to show that the problem of the functional activity of calcium is really concerned with the various forms in which calcium may exist, and the relationships of the different forms involved. Apparently, calcification does not bear a simple relationship to the calcium of the blood. In other cases of calcium disturbance, the rise or fall of total calcium in serum is but a very poor index of what may really be happening. With the acquisition of new knowledge concerning the physiological behavior of calcium in the organism, it has become more and more apparent that we are concerned with several different forms of calcium, which may or may not be in equilibrium with each other, or exert influence upon each other.

The conception that the biological importance of calcium in cellular activity may be attributed to ionic calcium, is of fairly recent origin. This appreciation of the possible importance of the calcium ion in physiological systems has been the natural result of the development of the theory of solutions from theoretical considerations concerning the influence of the electronic environment upon the properties of ions in general. In recent years, various methods have been employed in an effort to accomplish a difficult task, the measurement of calcium ion concentrations in liquid systems in general, and in serum and biological fluids in particular. Many have attempted to obtain these values by the analysis of ultrafiltrates, some by electrometric measurements with amalgam electrodes (Neuhausen

and Marshall, 1922), some by colorimetric methods, and others by calculation from experiments with difficultly soluble salts (Rona and Takahashi, 1911), (Kugelmass and Shohl, 1923–24), (Holt and collaborators, 1925, *a*, *b*). The latter method is the one which, in connection with the theoretical considerations outlined in the following, has been adopted for these studies.

Within the last few years a conception of ionic behavior has entered biological investigations, which relates the environment about the ions to the activity of the ions. Since this conception seems to be fruitful in the interpretation of studies involving electrolyte behavior generally, a brief review of the more important features will be given.

The failure of strong electrolytes to conform to the behavior predicted by the mass law, led G. N. Lewis in 1907 to propose a new thermodynamic concept called the *activity*, related to *fugacity* as concentration is to pressure; namely,

$$\text{fugacity} = RT \text{ activity}.$$

The fugacity may be regarded as the vapor pressure of a solute corrected for all deviations from the gas law, and hence is an accurate measure of the tendency of a substance to escape from its surroundings. In a similar way, by defining the activity of a substance as the ratio between the fugacity f of a substance in some given state, and its fugacity f^o in some other chosen standard state, we have a quantity which is an accurate measure of the escaping tendency of a substance expressed in terms of concentration units which have been corrected for all deviations from the gas laws. By using these new units corrected for deviations of the substance in the gaseous state, from the behavior of an ideal gas, one may apply them to any system, and yet obtain relationships which conform to those derived on the supposition that the gas laws are being obeyed. "Thus when stated in terms of fugacities or activities the law of mass action has a universal validity" (La Mer, 1925). However, since many substances are non-volatile, it has been more convenient to replace the absolute activity by the relative activity, for which the standard state of reference is defined in terms either of the pure liquid or of the pure solid state instead of the attenuated gaseous state.

In accordance with these principles, we have the relationship

$$\gamma = \frac{\alpha}{c} \tag{1}$$

where c is the stoichiometric molal concentration, α is the activity concentration, and γ is the activity coefficient, which, in dilute solutions, may be regarded as the thermodynamic degree of dissociation. In the case of solutions, the standard state is conveniently taken so that at infinite dilution of the solute, γ becomes unity, and all the gas laws are obeyed. Hence α will be equal to c at infinite dilution. Although the fugacity and activity of a solute at infinite dilution are proportional by Henry's law to the mol fraction N, one may still use molality units c as proportional to N in

dilute solutions. All of the data in these papers have been calculated on the molality basis; *i.e.*, in mols per kilo of H_2O.

Using the concept of activity, one may write for a strong electroyte such as NaCl,

$$\frac{\alpha_+ \times \alpha_-}{\alpha_o} = K \tag{2}$$

which is the mass law equation involving the thermodynamic concentrations or activities. The activity is related to the molal concentration so that

$$\alpha_+ = \gamma_+ c_+$$

$$\alpha_- = \gamma_- c_-$$

$$\alpha_o = \gamma_o c_o$$

$$\text{therefore,} \quad \frac{\gamma_+ c_+ \times \gamma_- c_-}{\gamma_o c_o} = K \tag{3}$$

This is the thermodynamic mass law equation for dissociated electrolytes where subscripts +, −, and o denote positive ion, negative ion, and salt, respectively.

Lewis and Randall (1923), studying activity coefficients in mixtures of different valence types, made the generalization that "*in dilute solutions*, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength." The ionic strength is related to the stoichiometric molality in the following manner:

Let μ = ionic strength

and $c_1\ c_2\ c_3 \ldots . c_n$ = molal concentration of ions present

$z_1\ z_2\ z_3 \ldots . z_n$ = valence of the ions,

then

$$\mu = \tfrac{1}{2}\ (c_1 z_1^2 + c_2 z_2^2 + c_3 z_3^2 \ldots . + c_n z_n^2) \tag{4}$$

Thus the concentration is related to the number and valence of ions present as they alter the electronic environment of a solution. It should be pointed out, however, that this is a limiting law, and serves only as a first approximation in solutions where individual ion properties are not significant.

The activity of a solute may be calculated from the vapor pressure of the solute, from the distribution of a solute between two solvents, from measurements of electromotive force, from freezing point data, and from solubility measurements. The solubility of slightly soluble salts in solutions of other salts has been utilized by many physical chemists, *e.g.*, Lewis, Brönsted, Noyes, Bray, Harkins, and their collaborators, to calculate the activity coefficients of the slightly soluble salts in the solution. It is this method which has been utilized in these studies.

At constant temperature, when a salt in solution is in equilibrium with the solid solute, it follows as a thermodynamic necessity that the activity of the salt in the solution is fixed and equal to the activity of the solid solute. No change in the solvent such as would be produced by addition of electrolytes or non-electrolytes can change that activity. If the activity coefficient of the salt in solution is decreased, due to a change in the solvent, more of the salt will go into solution until the activity in the solution and the solid state are equal. Hence

$$\gamma_o s_o = \gamma_1 s_1 = \gamma_2 s_2 \ldots\ldots\ldots \tag{5}$$

where s_o refers to solubility in pure water, and s_1 and s_2 refer to solubility in other solvents.

$$\frac{\gamma_o}{\gamma_1} = \frac{s_1}{s_o} \qquad \text{or} \quad -\log \gamma_1 = p\gamma_1 = \log \frac{s_1}{s_o} - \log \gamma_o \tag{6}$$

Setting γ_o arbitrarily equal to unity,

$$-\log \gamma_1 = p\gamma_1 = \log \frac{s_1}{s_o} \tag{7}$$

If a salt A_{ν_1}, B_{ν_2} dissociates so that $A_{\nu_1} B_{\nu_2} \rightleftarrows \nu_1 A + \nu_2 B$ at equilibrium, the following relationship must hold true:

$$(\alpha A^+)^{\nu_1} \times (\alpha B^-)^{\nu_2} = K_{s.p.} \tag{8}$$

where αA^+ and αB^- denote the activities of the ions, ν_1 and ν_2 are the number of ions of A^+ and B^- respectively, $+$ and $-$ are used regardless of valence, and $K_{s.p.}$ is the activity product or solubility product at ionic strength equal to zero. Substituting γc for α, where γ, c, and α refer to the ions, equation (8) becomes

$$(cA^+)^{\nu_1} \times (\gamma A^+)^{\nu_1} \times (cB^-)^{\nu_2} \times (\gamma B^-)^{\nu_2} = K_{s.p.} \tag{9}$$

or using the conventional symbol [ ] for concentration c,

$$[A^+]^{\nu_1} (\gamma A^+)^{\nu_1} \times [B^-]^{\nu_2} (\gamma B^-)^{\nu_2} = K_{s.p.} \tag{10}$$

By definition, the activity coefficient of a salt is the geometrical mean of the activity coefficients of its ions. Hence

$$\gamma A_{\nu_1} B_{\nu_2} = \sqrt[\nu_1 + \nu_2]{(\gamma A^+)^{\nu_1} \times (\gamma B^-)^{\nu_2}} \tag{11}$$

$$= (\gamma A^+)^{\frac{\nu_1}{\nu_1 + \nu_2}} \times (\gamma B^-)^{\frac{\nu_2}{\nu_1 + \nu_2}}$$

Logarithmically equation (11) becomes

$$\log \gamma A_{\nu_1} B_{\nu_2} = \frac{\nu_1}{\nu_1 + \nu_2} \log \gamma A^+ + \frac{\nu_2}{\nu_1 + \nu_2} \log \gamma B^- \tag{12}$$

Equation (10) may be written in the form

$$[A^+]^{\nu_1} \times [B^-]^{\nu_2} \times (\gamma A_{\nu_1} B_{\nu_2})^{\nu_1 + \nu_2} = K_{s.p.} \tag{13}$$

$$[A^+]^{\nu_1} \times [B^-]^{\nu_2} = \frac{K_{s.p.}}{(\gamma A_{\nu_1} B_{\nu_2})^{\nu_1 + \nu_2}} = K'_{s.p.} \tag{14}$$

We have found it convenient to transform equation (14) into its logarithmic form

$$\nu_1 \log [A^+] + \nu_2 \log [B^-] = \log K_{s.p.} - (\nu_1 + \nu_2) \log \gamma A_{\nu_1} B_{\nu_2} = \log K'_{s.p.} \tag{15}$$

As a further simplification, we have uniformly employed the symbol pX to designate $-\log [X^+]$. Equation (15) accordingly becomes

$$\nu_1 \, pA^+ + \nu_2 \, pB^- = pK_{s.p.} - (\nu_1 + \nu_2) \, p\gamma A_{\nu_1} B_{\nu_2} = pK'_{s.p.} \tag{16}$$

$$pK_{s.p.} - pK'_{s.p.} = (\nu_1 + \nu_2) \, p\gamma A_{\nu_1} B_{\nu_2} = \Delta \, pK_{s.p.} \tag{17}$$

$$\text{or } p\gamma A_{\nu_1} B_{\nu_2} = \frac{\Delta pK_{s.p.}}{\nu_1 + \nu_2} \tag{18}$$

Equation (18) is thus identical with that derived by Brönsted and La Mer (1924) by substituting $\sqrt[\nu]{K_{s.p.}}$ for s in equations (5), (6), and (7).

Debye and Hückel (1923), from purely physical considerations, employing the theory of complete dissociation of strong electrolytes first advanced by Bjerrum (1909), have recently elaborated a theory which accounts for the behavior of these substances by taking into consideration the electrical forces at play between the ions. Their mathematical treatment has been further simplified and applied to salts of higher valence type by Brönsted and La Mer, who found that the activity coefficient was related to the ionic strength of the solution by the equation

$$-\log \gamma_{salt} = \alpha' z_1 \, z_2 \sqrt{\mu} \tag{19}$$

where α' is a universal constant depending among other things on the dielectric constant of the medium, and z_1 and z_2 refer to the valences of the two ions of the salt. The addition of a correction term $\beta\mu$ enables one to use the equation for higher ionic strengths. More recently, Hückel (1925), developing the theory advanced by Milner and by Debye and Hückel, has derived a general working equation of the form

$$-\log \gamma_{salt} = \frac{B z_1 z_2 \sqrt{\mu}}{1 + A\sqrt{\mu}} + C\mu \tag{20}$$

In this equation A, B, and C denote constants. This equation takes into consideration the effect of the ionic environment, the size of the ions, and the dielectric properties of the solution. Since the concen-

trations of the solutions used in our experiments were less than 0.2 molal, we have neglected the possible deviation in the dielectric properties of the solutions with changing ionic strength. The equation used in these papers to relate the activity coefficient of the salts studied to the ionic strength has been of the general form

$$(21) \qquad -\log \gamma_{salt} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}}$$

For convenience in calculation, $Bz_1 z_2$ has been used as if it were one constant B.

These theoretical considerations will be further elaborated and discussed in connection with the experimental work as it is presented in later sections and papers. These sections given in order will contain a presentation of the technique used in the work, the calculations used, and the constants involved. Studies of the solubility of calcium carbonate and tertiary calcium phosphate, singly and together, under various conditions, will then follow.

Experimental Technique.

Analytical Methods.

Calcium.—Total calcium was determined by the method of Kramer and Tisdall (1921) slightly modified.[1] It was found that washing the precipitated and centrifuged calcium oxalate three times with 3 cc. of 2 per cent ammonia water, without stirring, gave results within 2 per cent of theoretical on solutions of known calcium content. Titrations were made with N/100 or N/200 $KMnO_4$ using 1 to 5 cc. samples according to the calcium content of the solution analyzed. The permanganate factor was redetermined in every experiment by means of standard sodium oxalate.

Clark and Collip (1925) have demonstrated that the conditions necessary for obtaining theoretical results with this and similar methods are purely empirical. Kramer and Tisdall, later Tisdall (1923) and Clark and Collip have all endeavored to work under

[1] Some of the later determinations were checked by a gasometric method developed by Dr. Van Slyke, as yet unpublished. The washed precipitated calcium oxalate is dissolved in sulfuric acid as usual and transferred quantitatively into the Van Slyke manometric gas analyzer (Van Slyke and Neill, 1924). The oxalic acid is oxidized with an excess of permanganate under the proper conditions, and the yield of carbon dioxide, which is quantitative, is taken as a measure of the calcium in the precipitate.

conditions such that the combined error due to solubility of calcium oxalate, and the retention of ammonium oxalate after washing, might be a minimum. As pointed out by the latter authors, any deviation in an arbitrary procedure will cause corresponding differences in results. Clark and Collip's modification, although quite satisfactory, was abandoned because of loss of precipitate when tubes were used which were not narrow enough at the bottom. In agreement with their findings, the original Kramer-Tisdall method gave low results. Some comparative results obtained by using different methods in determining the calcium content of a known solution are given below. Frequent determinations on the above solution, alone and when added to serum, served as a check on the accuracy of the method and the technique of the individual performing the analysis.

Determinations of Ca Content, by Different Methods, of an Aqueous Solution Containing 2.50 mM Ca per Liter.

Clark and Collip.	Kramer and Tisdall.	
	With stirring.	Without stirring.
2.48	2.32	2.50
2.48	2.32	2.51
2.46	2.32	2.51
2.53	2.35	2.52

Hydrogen Ion.—The $p\alpha_{H^+}$ of salt solutions and biological fluids was determined in the various ways indicated in the tables of experimental results: *e*, electrometric; *c*, colorimetric; *g*, gasometric results. Electrometric determinations were made in a thermostat maintained at 38 ± 1°. The temperature of the reaction liquid was read to 38 ± 0.2° in the Clark-Cullen electrode vessel of 2 cc. capacity. A thermometer in the electrode vessel, as suggested by Cullen, permitted accurate temperature control of the liquid under investigation. The gas chain employed was the following.

Hg	HgCl	Saturated KCl	Saturated KCl bridge	Unknown solution	H_2	Pt

Pure mercury was prepared according to Hulett (1905) by redistilling five times. Calomel was prepared electrolytically from the purified mercury and redistilled hydrochloric acid. Merck's analytical KCl was used in the saturated calomel electrode and salt bridge. The hydrogen used was found by analysis to be 99.5 per cent pure. Whenever CO_2-containing solutions were being measured, the electrode was first flushed and filled with a mixture of hydrogen and CO_2, the latter gas being at the same tension as that of the CO_2 in the liquid. Usually the gas phase, with which the unknown had been equilibrated, was itself run from the large saturation tonometer into the electrode vessel, by displacement with mercury. Electrodes were made of heavy platinum foil. Before use in each experiment, they were freshly coated with platinum black. Platinic chloride for plating electrodes was prepared from pure platinum.

The standard used was N/10 HCl made from Hulett's (1909) constant boiling acid, twice redistilled. The strength of the N/10 HCl was confirmed by gravimetric analyses which indicated a chloride content within 0.1 per cent of theoretical. The $p\alpha_{H^+}$ of this acid, on the basis of Lewis' (1923) value (p. 382) for the activity of H^+ ion in a solution of this ionic strength, was assumed to be 1.08 at 38° and 20°. No correction was made for liquid junction potential.

Values of $p\alpha_{H^+}$ of unknown solutions were calculated from the equation:

$$p\alpha_{H^+} = \frac{E \text{ (corrected to 1 atmosphere of dry hydrogen)} - e_o}{0.0001983\ T}.^2$$

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[2] It may be noted here, that $p\alpha_{H^+}$ referred to in these papers is identical with $p\alpha_{H^+}$ as used by Hastings and Sendroy (1925) and with the pH of Van Slyke, Hastings, Murray, and Sendroy (1925). However, this value is *not* identical with the $p\alpha_H$ of Sörensen and Linderström-Lang (1924), which is approximately 0.04 higher than ours, but corresponds to their pH, which is obtained by using the Bjerrum extrapolation for liquid junction potential, and assigning to N/10 HCl a value based on conductivity measurements. No correction for liquid junction potentials between saturated KCl and either N/10 HCl or our unknown solutions has been attempted. If in the future the difference between these diffusion potentials is established our $p\alpha_{H^+}$ values are subject to a corresponding correction.

In many of the experiments, $p\alpha_{H^+}$ was determined colorimetrically on both salt solutions and serum, using bicolor standards and reading at 38°, according to the technique of Hastings and Sendroy (1924). From time to time, the color standards were checked against phosphate standards which in turn had been standardized electrometrically. Salt solutions were read *immediately* after sampling under oil for such solutions lose CO_2 and change their reaction very quickly. Colorimetric determinations on serum were accurate to $+0.05$ $p\alpha_{H^+}$, salt solutions to $+0.02$.[3]

Sometimes when a high degree of accuracy was required, or when the reaction was too acid or too alkaline to be read within the phenol red range, $p\alpha_{H^+}$ was indirectly determined from total CO_2 and CO_2 tension analyses, as outlined in the section on calculations.

CO_2 Tension.—The gas phase was analyzed in the Henderson-Haldane apparatus of 10 cc. capacity. The accuracy of these analyses was approximately 0.5 per cent.

CO_2 Content.—Total CO_2 was determined in the manometric gas apparatus described by Van Slyke and Neill (1924). These determinations were accurate to about 0.3 per cent.

H_2O Content.—Moisture determinations for converting data from molarity to molality basis were obtained by drying 1 or 2 cc. samples for about 15 to 20 hours in a Freas oven at 120°C. Weighings were made to 1 mg., and the error involved was not more than 0.2 per cent.

[3] The bicolor standards referred to, as described previously (Hastings and Sendroy, 1924), have values based on the comparison of such standards against electrometrically standardized M/15 Sörensen phosphate standards. However, experiments as yet unpublished indicate that the effect of ionic strength and valence type of salt on the activity of dyes is similar to their effect on other ions. In other words, the apparent dissociation constant of the dye changes with ionic strength and with different salts in the solution, thus behaving in conformity with the Debye-Hückel theory of strong electrolytes. In order to detect any possible difference in activity of phenol red in the M/15 phosphate solution and in artificial edema fluid, such as was usually employed, comparative results were obtained by electrometric and colorimetric determinations of the same solutions. Since the two methods agreed in all cases to within 0.01 $p\alpha_{H^+}$, it was decided that all readings on balanced salt solutions obtained with the bicolor standards could be considered reliable.

XUM

Pure mercury was prepared according to Hulett (1905) by redistilling five times. Calomel was prepared electrolytically from the purified mercury and redistilled hydrochloric acid. Merck's analytical KCl was used in the saturated calomel electrode and salt bridge. The hydrogen used was found by analysis to be 99.5 per cent pure. Whenever CO_2-containing solutions were being measured, the electrode was first flushed and filled with a mixture of hydrogen and CO_2, the latter gas being at the same tension as that of the CO_2 in the liquid. Usually the gas phase, with which the unknown had been equilibrated, was itself run from the large saturation tonometer into the electrode vessel, by displacement with mercury. Electrodes were made of heavy platinum foil. Before use in each experiment, they were freshly coated with platinum black. Platinic chloride for plating electrodes was prepared from pure platinum.

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XUM

Phosphorus.—Determinations of the phosphorus in salt solutions and serum were made according to the method of Benedict and Theis (1924), an improvement on the Briggs (1922) modification of the original Bell and Doisy (1920) colorimetric method. Stanford and Wheatley (1925) have recently furnished proof of the validity of the principle upon which the method depends, and report that there is a direct proportionality between the color produced and the phosphate present, only when the method is done under certain conditions. Inasmuch as Benedict and Theis also confirm Buell's (1923) finding that there is no appreciable amount of acid-soluble organic phosphorus in serum which is hydrolyzed by concentrated sulfuric acid, a determination by this method, even in serum, can most probably be taken to represent only the inorganic phosphorus, hence the total phosphate. In our experiments we have calculated our phosphate results on this assumption. We have confirmed Stanford and Wheatley in that accurate results can only be obtained when total acidity, concentration of all reagents, and temperature are strictly uniform in standards and unknown solutions.

The reading of the solution and standard must not differ by more than 20 per cent in order to obtain a reading which is within 5 per cent of theoretical. In serum analyses, the precipitation of proteins in a solution diluted only five times may very well lead to still greater error. However, this is unavoidable in such cases where the extremely low phosphate content of the solution analyzed makes it necessary to have as concentrated a filtrate as possible, in order to be able to read the slight coloration produced.

Saturation Procedure.

Salt solutions approximating quantitatively and qualitatively the content of edema fluid and other body fluids as analyzed by Salvesen (1923) were made up as indicated in the protocols. Standard solutions of NaCl, KCl, $MgCl_2$, $CaCl_2$, $NaHCO_3$, K_2SO_4, and Na_2HPO_4-KH_2PO_4 of known composition were prepared. Quantities of these solutions required to give the desired final concentration of each of these salts were accurately pipetted into a 200 cc. volumetric flask. Before the $CaCl_2$ was introduced, 125 cc. of water saturated with CO_2 were added to the flask to

prevent premature precipitation of the calcium as carbonate or phosphate. The solution was then made up to 200 cc. and transferred to the saturating tonometer system previously described by Austin and collaborators (1922). About 50 cc. of this salt solution were introduced into either a small tonometer or a 50 cc. narrow mouth centrifuge tube, about 1 per cent in weight of solid added, and then connection made with rubber tubing to a larger tonometer of from 300 to 400 cc. capacity.

The liquid with solid was clamped off from the large tonometer, which was washed out three times with either air or hydrogen, depending on whether or not $p\alpha_{H^+}$ was to be measured electrometrically; the tension of CO_2 required to give the desired final reaction was introduced, and then air or hydrogen run in to 1 atmosphere pressure. The liquid phase was then allowed to run into the large tonometer, the whole being placed in a water bath maintained at 38 $\pm$ 0.05° and rotated for 20 minutes. This procedure was repeated three more times, in order to eliminate excess CO_2 and so approach the desired final tension. At the fourth saturation, air or hydrogen was run in only to within 80 mm. of atmospheric pressure. The next day, after rotation from 18 to 24 hours, the whole was equilibrated to the atmosphere, then liquid and solid were separated from the gas phase. When saturation was carried on for several days, the CO_2 tension was adjusted several times during the saturation period, and then, finally, about 20 hours or so before being analyzed.

When the CO_2 tension was desired, samples of gas were taken in small 70 cc. Barcroft tubes or else sampled directly from the large tonometer. When centrifuge tubes were used for the liquid and solid, these were quickly placed in a centrifuge after covering with oil and rubber stoppers, or else paraffin, to prevent loss of CO_2. After centrifugation, the clear supernatant fluid was transferred to small containers over mercury. When small tonometers were used directly, the solid phase was removed by filtration through small glass tubes containing cotton.

Early in the work, when solutions were kept in contact with the solid salt, a constant temperature of 38° in centrifuging was maintained. However, it soon became apparent that the difference in solubility of calcium carbonate or phosphate between room and body temperature during the period between the end of

saturation and completion of all analyses, was within the limit of error of the methods of analysis. When absorbent cotton was

TABLE I.

Effect of Variation within the Known Experimental Limit of Error of Any of the Factors in Calculation of $pK'_{s.p.}$ of $CaCO_3$ and $Ca_3(PO_4)_2$.

$p_{\alpha}H^+$	$[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[CO_3]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa^{++}	$pCO_3^=$	$pPO_4^{\equiv}$	$pK'_{s.p.}$ $CaCO_3$	$pK'_{s.p.}$ $Ca_3(PO_4)_2$
7.43	0.28	30.32	0.293	3.55	3.90	7.95	7.45	26.55
7.38	0.28	30.32	0.293	3.55	3.95	8.01	7.50	26.67
−0.05							+0.05	+0.12
7.43	0.28	30.32	0.293	3.55	3.90	7.95	7.45	26.55
7.43	0.266	30.32	0.293	3.58	3.90	7.95	7.48	26.64
	−5 per cent.						+0.03	+0.09
7.43	0.28	30.32		3.55	3.90		7.45	
7.43	0.28	30.22		3.55	3.90		7.45	
		−0.3 per cent.					±0.00	
7.43	0.28		0.293	3.55		7.95		26.55
7.43	0.28		0.278	3.55		7.97		26.59
			−5 per cent.					+0.04

Summary.

±0.05							∓0.05	∓0.12
	±5 per cent.						∓0.03	∓0.09
		±0.3 per cent.					±0.00	
			±5 per cent.					∓0.04
Average error.....							±0.04	±0.08
Maximum "							±0.08	±0.25

used for filtration, it was thought that stray shreds of cotton might affect the determination of the calcium. To test this,

comparisons were made on clear solutions, some containing a few shreds, and others more cotton, during the heating with normal sulfuric acid and subsequent titration. The presence of cotton in these solutions within the time required to dissolve the calcium oxalate and to titrate, had no effect in reducing the permanganate.

The solid $CaCO_3$ and $Ca_3(PO_4)_2$ used were of the highest purity obtainable. Before use in these experiments, both were washed five times with distilled water, then dried at 110°.

Table I indicates the limit of accuracy attainable with these methods, and the effect of errors in determining the values $pK'_{s.p.}$ $CaCO_3$ and $pK'_{s.p.}$ $Ca_3(PO_4)_2$.

Symbols.

The symbols below have been used in this and the following papers.

$\alpha_{Ca^{++}}$ = activity of Ca^{++} ion.
$\gamma_{Ca^{++}}$ = " coefficient of Ca^{++} ion.
$[Ca^{++}]$ = concentration of Ca^{++} ion in mols per kilo of H_2O.
$[Ca]$ = " " total Ca " " " " " " "
$[CaP]$ = " " Ca bound by protein in mols per kilo of H_2O.
$[CaX]$ = concentration of Ca not in ionized form in mols per kilo of H_2O.
μ = ionic strength.
ν = number of ions of a salt.
pCa^{++} = negative logarithm of $[Ca^{++}]$.
$p\gamma_{Ca^{++}}$ = " " " $\gamma_{Ca^{++}}$.
K = activity dissociation constant of an acid.
K′ = stoichiometric dissociation constant of an acid.
pK = negative logarithm of K.
$pK_{s.p.}$ $CaCO_3$ = $p[\alpha_{Ca^{++}} \times \alpha_{CO_3^{=}}]$.
$pK'_{s.p.}$ $CaCO_3$ = $p([Ca^{++}] \times [CO_3^{=}])$.
$\alpha^{\circ}_{H_2O}$ = solubility coefficient of CO_2 in pure water.
$\alpha^{\circ}_{CO_2}$ = " " " " " salt solution and serum.
pCO_2 = CO_2 tension in mm. of Hg.
f_3 = $pCO_2 : [CO_2]$.
f_2 = $[H_2CO_3] : [CO_2]$.
f_1 = $[HCO_3^-] : [CO_2]$.
f = $[CO_3^{=}] : [CO_2]$.
mM = millimols = M $\times 10^3$.

Calculations.

Calculation of $[CO_3^{=}]$ *Ion Concentration from Total* CO_2 *Content and* $p\alpha_{H^+}$.

From the mass law,

$$\frac{\alpha_{H^+} \times \alpha_{HCO_3^-}}{\alpha_{H_2CO_3}} = K_1 \tag{22}$$

$$\frac{\alpha_{H^+} \times \gamma_{HCO_3^-} \times [HCO_3^-]}{\gamma_{H_2CO_3} \times [H_2CO_3]} = K_1 \tag{23}$$

$$\frac{\alpha_{H^+} \times [HCO_3^-]}{\gamma_{H_2CO_3} \times [H_2CO_3]} = \frac{K_1}{\gamma_{HCO_3^-}} = K_1' \tag{24}$$

$$\frac{\alpha_{H^+} \times [HCO_3^-]}{[H_2CO_3]} = \frac{K_1 \times \gamma_{H_2CO_3}}{\gamma_{HCO_3^-}} = K_1'' \tag{25}$$

However, K_1'' is a *constant* only under conditions in which $\gamma_{H_2CO_3}$ and $\gamma_{HCO_3^-}$ are constant.

In like manner $\frac{\alpha_{H^+} \times [CO_3^{=}]}{[HCO_3^-]} = \frac{K_2 \times \gamma_{HCO_3^-}}{\gamma_{CO_3^=}} = K_2'$ (26)

$$\text{Total } [CO_2] = [H_2CO_3] + [HCO_3^-] + [CO_3^{=}] \tag{27}$$

By equation (26)

$$[HCO_3^-] = \frac{\alpha_{H^+} \times [CO_3^{=}]}{K_2'} \tag{28}$$

By equations (25) and (28),

$$[H_2CO_3] = \frac{\alpha_{H^+} \times [HCO_3^-]}{K_1''} = \frac{(\alpha_{H^+})^2 \times [CO_3^{=}]}{K_1'' K_2'} \tag{29}$$

Therefore, equation (27) can be written

$$\text{Total } [CO_2] = \frac{(\alpha_{H^+})^2 \times [CO_3^{=}]}{K_1'' K_2'} + \frac{\alpha_{H^+} \times [CO_3^{=}]}{K_2'} + [CO_3^{=}] \tag{30}$$

$$\text{Total } [CO_2] = [CO_3^{=}] \times \left[\frac{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'}{K_1'' K_2'}\right] \tag{31}$$

$$[CO_3^{=}] = \frac{\text{Total } [CO_2] \times K_1'' K_2'}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'} \tag{32}$$

TABLE II.

Factors for the Calculation of $[CO_3^{=}]$ and $[HCO_3^{-}]$ from the Total $[CO_2]$ Content in Salt Solutions of $\mu = 0.152$ and in Serum.

$pK_1'' = 6.13$, $pK_2' = 9.79$

$p\alpha_{H^+}$	f where $f \times [CO_2] = [CO_3^{=}]$	f_1 where $f_1 \times [CO_2] = [HCO_3^{-}]$	$p\alpha_{H^+}$	f where $f \times [CO_2] = [CO_3^{=}]$	f_1 where $f_1 \times [CO_2] = [HCO_3^{-}]$
6.0	0.00007	0.426	7.11	0.00189	0.904
6.1	0.00010	0.478	7.12	0.00194	0.906
6.2	0.00014	0.540	7.13	0.00199	0.908
6.3	0.00019	0.597	7.14	0.00204	0.909
6.4	0.00027	0.650	7.15	0.00209	0.911
6.5	0.00036	0.700	7.16	0.00214	0.913
6.6	0.00048	0.746	7.17	0.00219	0.914
6.7	0.00064	0.788	7.18	0.00224	0.916
6.80	0.00084	0.823	7.19	0.00230	0.918
6.81	0.00086	0.826	7.20	0.00236	0.919
6.82	0.00089	0.830	7.21	0.00242	0.921
6.83	0.00091	0.833	7.22	0.00248	0.923
6.84	0.00094	0.836	7.23	0.00254	0.924
6.85	0.00096	0.839	7.24	0.00260	0.926
6.86	0.00099	0.842	7.25	0.00266	0.927
6.87	0.00101	0.845	7.26	0.00273	0.928
6.88	0.00104	0.848	7.27	0.00280	0.930
6.89	0.00107	0.851	7.28	0.00287	0.932
6.90	0.00110	0.854	7.29	0.00294	0.933
6.91	0.00113	0.857	7.30	0.00301	0.934
6.92	0.00116	0.860	7.31	0.00309	0.936
6.93	0.00119	0.863	7.32	0.00317	0.937
6.94	0.00122	0.865	7.33	0.00325	0.938
6.95	0.00125	0.867	7.34	0.00333	0.939
6.96	0.00129	0.870	7.35	0.00341	0.940
6.97	0.00132	0.872	7.36	0.00349	0.941
6.98	0.00135	0.875	7.37	0.00358	0.942
6.99	0.00139	0.877	7.38	0.00367	0.943
7.00	0.00142	0.880	7.39	0.00376	0.944
7.01	0.00146	0.882	7.40	0.00385	0.945
7.02	0.00150	0.884	7.41	0.00394	0.946
7.03	0.00154	0.887	7.42	0.00404	0.947
7.04	0.00158	0.889	7.43	0.00414	0.948
7.05	0.00162	0.891	7.44	0.00424	0.949
7.06	0.00166	0.893	7.45	0.00434	0.950
7.07	0.00171	0.896	7.46	0.00444	0.951
7.08	0.00175	0.898	7.47	0.00455	0.952
7.09	0.00179	0.900	7.48	0.00466	0.953
7.10	0.00184	0.902			

TABLE II—*Concluded.*

$p\alpha_{H^+}$	f where $f \times [CO_3] = [CO_3^=]$	f_1 where $f_1 \times [CO_3] = [HCO_3^-]$	$p\alpha_{H^+}$	f where $f \times [CO_3] = [CO_3^=]$	f_1 where $f_1 \times [CO_3] = [HCO_3^-]$
7.49	0.00477	0.954	7.71	0.00802	0.967
7.50	0.00489	0.954	7.72	0.00821	0.967
7.51	0.00501	0.955	7.73	0.00841	0.968
7.52	0.00513	0.956	7.74	0.00861	0.968
7.53	0.00525	0.957	7.75	0.00881	0.968
7.54	0.00537	0.958	7.76	0.00901	0.969
7.55	0.00550	0.958	7.77	0.00922	0.969
7.56	0.00565	0.959	7.78	0.00943	0.969
7.57	0.00578	0.960	7.79	0.00965	0.970
7.58	0.00591	0.960	7.80	0.00988	0.970
7.59	0.00607	0.961	7.9	0.01249	0.9711
7.60	0.00621	0.961	8.0	0.01573	0.9712
7.61	0.00636	0.962	8.1	0.01979	0.9698
7.62	0.00651	0.963	8.2	0.02480	0.9669
7.63	0.00666	0.963	8.3	0.03112	0.9623
7.64	0.00681	0.964	8.4	0.03890	0.956
7.65	0.00697	0.964	8.5	0.04858	0.9474
7.66	0.00713	0.965	8.6	0.0604	0.9364
7.67	0.00730	0.965	8.7	0.0748	0.9227
7.68	0.00747	0.966	8.8	0.0921	0.9059
7.69	0.00765	0.966	8.9	0.1141	0.8844
7.70	0.00783	0.966	9.0	0.1394	0.8594

In a similar way

$$[HCO_3^-] = \frac{\text{Total } [CO_2] \times \alpha_{H^+} K_1''}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'} \tag{33}$$

Knowing K_1'', and K_2' for any particular solution, one may derive a set of values for each $p\alpha_{H^+}$, such that

$$f = \frac{K_1'' K_2'}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'} \tag{34}$$

and

$$f_1 = \frac{\alpha_{H^+} K_1''}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'} \tag{35}$$

where f and f_1 are really factors representing the percentage of total $[CO_3]$ in the form of $[CO_3^=]$ and $[HCO_3^-]$ respectively. Values for use with salt solutions at $\mu = 0.152$ and with serum, between $p\alpha_{H^+}$ 6.0 and 9.0 at 0.1 intervals, and for $p\alpha_{H^+}$ between 6.8 and 7.8 at 0.01 intervals are given in Table II.

Calculation of $p\alpha_{H^+}$ from Analyses of CO_2 Content and CO_2 Tension.

In experiments in which it was not possible to determine $p\alpha_{H^+}$ directly, this value was obtained indirectly by calculation from analyses of the total CO_2 content and the CO_2 tension. Expressing H_2CO_3 in terms of the CO_2 tension, pCO_2,

$$(36) \quad \alpha_{H_2CO_3} = \frac{\alpha^o_{H_2O} \times pCO_2}{760 \times 0.0224} = \frac{0.557 \times pCO_2}{760 \times 0.0224} = 0.0327\ pCO_2$$

$$(37) \quad [H_2CO_3] = \frac{\alpha^o_{CO_2} \times pCO_2}{760 \times 0.0224} = \frac{(0.557 - 0.103\ \mu + 0.36\ P) \times pCO_2}{760 \times 0.0224}$$

where, according to the notation in unpublished work of Van Slyke, Hastings, and Neill, $\alpha^o_{H_2O}$ and $\alpha^o_{CO_2}$ are the solubility coefficients of CO_2 in pure water, and in a salt- and protein-containing solution. μ has its usual significance and P indicates gm. of protein per gm. of H_2O in the equation. We can write equation (27) in the form

$$(38) \quad \text{Total } [CO_2] = f\ [CO_2] + f_1\ [CO_2] + f_2\ [CO_2]$$

where f, f_1, and f_2 refer to the percentage factor of the total $[CO_2]$ in the form of $[CO_3^=]$, $[HCO_3^-]$, and $[H_2CO_3]$ respectively. It has been shown how f and f_1 may be derived in equations (34) and (35). Hence f_2 may be found by difference, by

$$(39) \quad 1.00 - f - f_1 = f_2$$

By definition,

$$(40) \quad f_2\ [CO_2] = [H_2CO_3] = \frac{\alpha^o_{CO_2} \times pCO_2}{760 \times 0.0224}$$

For solutions of a *constant composition*, $\alpha^o_{CO_2}$ will be constant, and we may write

$$(41) \quad pCO_2 = \frac{760 \times 0.0224 \times f_2\ [CO_2]}{\alpha^o_{CO_2}} = f_3\ [CO_2]$$

where

$$(42) \quad f_3 = \frac{f_2 \times 760 \times 0.0224}{\alpha^o_{CO_2}}$$

By equations (39), (34), and (35)

$$(43) \quad \frac{K_1'' K_2' + \alpha_{H^+} K_1''}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'} + f_2 = 1.00$$

TABLE III.

Factors for the Calculation of $p\alpha_{H^+}$ from Total CO_2 Content and CO_2 Tension for Use in Serum.

$f_3 = \frac{pCO_2}{[CO_2]}$	$\alpha^{o}_{CO_2} = 0.565$
$p\alpha_{H^+}$	f_3
6.0	17.31
6.1	15.72
6.2	13.855
6.3	12.138
6.4	10.542
6.5	9.036
6.6	7.650
6.7	6.385
6.8	5.301
6.9	4.367
7.0	3.584
7.1	2.891
7.2	2.379
7.3	1.898
7.4	1.536
7.5	1.235
7.6	0.994
7.7	0.783
7.8	0.620
7.9	0.494
8.0	0.395
8.1	0.313
8.2	0.250
8.3	0.199
8.4	0.154
8.5	0.123
8.6	0.096
8.7	0.078
8.8	0.054
8.9	0.045
9.0	0.036

(44) $$f_2 = 1 - \frac{K_1\ K_2' + \alpha_{H^+} K_1''}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'}$$

(45) $$f_2 = \frac{(\alpha_{H^+})^2}{(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2'}$$

(46) $$\text{Hence, } f_3 = \frac{(\alpha_{H^+})^2 \times 760 \times 0.0224}{\alpha^o_{CO_2}[(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2']}$$

(47) $$\text{and } pCO_2 = \frac{\text{Total } [CO_2] \times (\alpha_{H^+})^2 \times 760 \times 0.0224}{\alpha^o_{CO_2}[(\alpha_{H^+})^2 + \alpha_{H^+} K_1'' + K_1'' K_2']}$$

Equation (47) expresses the relationship between the CO_2 tension, total CO_2, and α_{H^+}. In actual practice, no attempt was made to calculate $p\alpha_{H^+}$ according to equation (47). Having calculated f and f_1 at various $p\alpha_{H^+}$'s f_2 was found by difference, and then f_3, for the corresponding $p\alpha_{H^+}$ values. Then, by plotting f_3 against $p\alpha_{H^+}$ a curve was obtained for the relationship $f_3 = \frac{pCO_2}{\text{total } [CO_2]}$ at any desired $p\alpha_{H^+}$. By determining the CO_2 tension and total CO_2, f_3 values obtained gave the corresponding $p\alpha_{H^+}$. Corresponding values of f_3 and $p\alpha_{H^+}$ are given in Table III.

Inasmuch as $\alpha^o_{CO_2}$ is a variable depending on the composition of a solution, the relationships given will not always hold for the factors f_2 and f_3 as given in the foregoing. In order to calculate $p\alpha_{H^+}$ from total $[CO_2]$ and CO_2 tension, in *solutions of changing composition*, one proceeds as follows, using the $\alpha^o_{CO_2}$ which is the *appropriate one for that solution* whose $p\alpha_{H^+}$ is to be determined. Van Slyke, Hastings, and Neill have found (unpublished results) that $\alpha^o_{CO_2}$ (cc. of CO_2 per gm. of H_2O) varies according to the empirical formula

(48) $$\alpha^o_{CO_2} = 0.557 - 0.103\mu + 0.36P^4$$

where P = gm. of protein per gm. of H_2O.

The solutions for which $p\alpha_{H^+}$ was determined in the manner to be out-

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[4] As this material goes to press, it is now evident that recent work gives as a more accurate expression for the solubility of CO_2 in salt and protein solutions, the following equation

$$\alpha^o_{CO_2} = 0.554 - 0.104\mu + 0.36\ P.$$

Our results would not be significantly altered by calculating according to the above equation.

lined here, were never at a reaction higher than about 7.5. Hence the $[CO_3^{=}]$ ion concentration was so small that it could be neglected. Equation (25) therefore could be written

$$\frac{\alpha_{H^+} \times ([CO_2] - [H_2CO_3])}{[H_2CO_3]} = K_1'' \tag{49}$$

By using the proper value for $\alpha^o_{CO_2}$ in the solution under investigation, $[H_2CO_3]$ was calculated according to equation (37), and then $p\alpha_{H^+}$ found by converting equation (49) into the well known logarithmic form of the Henderson-Hasselbalch equation:

$$p\alpha_{H^+} = pK_1'' + \log \frac{[CO_2] - [H_2CO_3]}{[H_2CO_3]} \tag{50}$$

We may derive the actual working equation corresponding to equation (47) as follows:

$$\gamma_{H_2CO_3} = \frac{\alpha_{H_2CO_3}}{[H_2CO_3]} \tag{51}$$

Substituting the values for $\alpha_{H_2CO_3}$ and $[H_2CO_3]$ given in equations (36) and (37)

$$\gamma_{H_2CO_3} = \frac{0.557}{0.557 - 0.103\mu + 0.36P} = \frac{1}{1 - 0.185\mu + 0.65P} \tag{52}$$

$$[H_2CO_3] = 0.0327\ pCO_2\ (1 - 0.185\mu + 0.65P) \tag{53}$$

Also by equation (36)

$$p\alpha_{H_2CO_3} = 1.486 + p\ pCO_2 \tag{54}$$

$$p\alpha_{HCO_3^-} = p\gamma_{HCO_3^-} + p\ [HCO_3^-] \tag{55}$$

$$p\gamma_{HCO_3^-} = 0.5\sqrt{\mu} \tag{56}$$

Combining equations (27), (53), (54), (55), (56),

$$p\alpha_{HCO_3^-} = 0.5\sqrt{\mu} + p\{[CO_2] - [CO_3^{=}] - 0.0327\ pCO_2(1 - 0.185\mu + 0.65P)\} \tag{57}$$

But since $pK_1 = 6.33$ at 38°, equation (22) may be written in the logarithmic form

$$p\alpha_{H^+} = 7.816 - 0.5\sqrt{\mu} + p\ p\ CO_2 - p\{[CO_2] - [CO_3^{=}] - 0.0327\ pCO_2 (1 - 0.185\mu + 0.65P)\} \tag{58}$$

In salt solutions containing no protein and in which the $[CO_3^=]$ concentration is negligible, equation (58) simplifies into

$$(59)\quad p\alpha_{H^+} = 7.816 - 0.5\sqrt{\mu} + p\,pCO_2 - p\{[CO_2] - pCO_2\,(0.0327 - 0.00605\mu)\}$$

In serum of average salt and protein composition where $\mu = 0.16$ and $P = 0.07$ gm. per gm. of H_2O, equation (58) becomes

$$(60)\quad p\alpha_{H^+} = 7.616 + p\,pCO_2 - p\{[CO_2] - 0.0332\,pCO_2\}$$

When the $[CO_3^=]$ is not neglible, *i.e.* when the $p\alpha_{H^+}$ is greater than 8.0, the $p\alpha_{H^+}$ is first approximated by equation (59) or (60), then the $[CO_3^=]$ is estimated by determining the value of f for the approximated $p\alpha_{H^+}$. The CO_2 content when multiplied by this value gives the $CO_3^=$ concentration. This value for $[CO_3^=]$ is then substituted in equation (58) and the $p\alpha_{H^+}$ again calculated. Since most $p\alpha_{H^+}$ values are less than 8.0, however, it is not often necessary to apply equation (58).

Constants Used in CO_2 Calculations.

The values of the first and second dissociation constants and activity coefficients used in the calculations were taken from the determinations of Hastings and Sendroy (1925). The equations given by them for these constants are

$$(61)\quad pK_1' = 6.33 - 0.5\sqrt{\mu}$$

$$(62)\quad pK_2' = 10.22 - 1.1\sqrt{\mu}$$

For the balanced salt solutions used, the values calculated for an approximate ionic strength of $\mu = 0.152$, were $pK_1' = 6.13$, $pK_1'' = 6.14$. Van Slyke, Hastings, Murray, and Sendroy (1925) found $pK_1' = 6.13$, $pK_1'' = 6.14$ for horse serum, while Hastings and Sendroy (unpublished results) have found $pK_1' = 6.12$ and $pK_1'' = 6.13$ in human serum. Therefore, in order to simplify the calculations, $pK_1'' = 6.13$ was used in experiments with the serum of horse and humans and in salt solutions of approximate $\mu = 0.152$. For the same ionic strength, $pK_2' = 9.79$. Hence $K_1'' = 7.41 \times 10^{-7}$ and $K_2' = 1.62 \times 10^{-10}$. For $\alpha^o_{H_2O}$ the value used was 0.557. In salt solutions and serum the variation of $\alpha^o_{CO_2}$ was calculated according to equation (48). These solubility coefficients were derived without attempting to differentiate between the two reactions.

$$\underset{\text{Gas.}}{CO_2} \rightleftarrows \underset{\text{Dissolved.}}{CO_2} \quad \text{and} \quad CO_2 + H_2O \rightleftarrows \underset{\text{Dissolved.}}{H_2CO_3}$$

Experimental Results.

Variation in Solubility of $CaCO_3$ with Varying Ionic Strength.

The solubility of $CaCO_3$ has been determined in salt solutions, the ionic strength of which ranged from $\mu = 0.024$ to $\mu = 0.208$. Save for small variations in bicarbonate concentration, the ionic

TABLE IV.

Solubility of $CaCO_3$ at 38° in Salt Solutions of Varying Ionic Strength.

Experiment No.	μ	$\sqrt{\mu}$	$p\alpha_{H^+}$	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^{=}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCO_2 =	pCa^{++}	pK'a.p. $CaCO_3$ determined.	pK'a.p. $CaCO_3$ calculated.
1	0.024	0.156	7.02 c	18.86	0.0152	0.764	4.82	3.12	7.94	7.96
2	0.031	0.176	7.22 c	24.56	0.0348	0.372	4.46	3.43	7.89	7.90
3	0.050	0.224	7.07 c	18.93	0.0205	0.865	4.69	3.06	7.75	7.76
4	0.076	0.276	7.10 c	19.02	0.0253	0.916	4.60	3.04	7.64	7.64
5	0.082	0.286	7.27 c	25.07	0.0541	0.423	4.27	3.37	7.64	7.61
6	0.102	0.320	7.00 c	20.00	0.0231	1.208	4.64	2.92	7.56	7.54
7	0.108	0.329	7.28 c	25.68	0.0621	0.474	4.21	3.32	7.53	7.52
8	0.129	0.359	7.52 g	9.86	0.0506	0.706	4.30	3.15	7.45	7.46
9	0.130	0.361	7.27 g	11.06	0.0310	1.134	4.51	2.95	7.46	7.46
10	0.130	0.361	7.37 g	11.56	0.0414	0.958	4.38	3.02	7.40	7.45
11	0.131	0.362	7.16 g	12.50	0.0267	1.482	4.57	2.83	7.40	7.45
12	0.132	0.363	7.17 g	14.05	0.0308	1.330	4.51	2.88	7.39	7.45
13	0.133	0.365	7.41 c	25.40	0.0941	0.494	4.03	3.31	7.34	7.44
14	0.137	0.370	7.16 g	18.80	0.0402	1.008	4.40	3.00	7.40	7.44
15	0.158	0.398	7.38 c	25.51	0.0963	0.494	4.02	3.31	7.33	7.38
16	0.177	0.420	7.30 c	18.22	0.0594	1.040	4.23	2.98	7.21	7.34
17	0.177	0.420	8.35 g	24.87	0.870	0.050	3.06	4.30	7.36	7.34
18	0.177	0.420	7.48 g	27.07	0.126	0.352	3.90	3.45	7.35	7.34
19	0.202	0.450	7.32 c	18.44	0.0683	1.04	4.17	2.98	7.15	7.29
20	0.208	0.455	7.46 c	23.70	0.1243	0.371	3.91	3.43	7.34	7.28

strength was increased by additions of NaCl. After 20 hours of saturation at 38° with solid $CaCO_3$, the liquid phase was analyzed for the CO_2 content, calcium content, and $p\alpha_{H^+}$. In some instances the CO_2 tension in the gas phase was also determined. From the CO_2 content and $p\alpha_{H^+}$ the $[CO_3^{=}]$ concentration was calculated. From the $[CO_3^{=}]$ concentration and the $[Ca^{++}]$ concentration

the $pK'_{s.p.}$ $CaCO_3$ was calculated. The ionic strength, μ, of the solution was calculated from the analyses of the solution at the end of the saturation period. The results of these experiments have been tabulated in Table IV and are graphically presented in Fig. 1. The smooth curve is the graphical representation of the equation

$$pK'_{s.p.}\ CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.61\sqrt{\mu}} \tag{63}$$

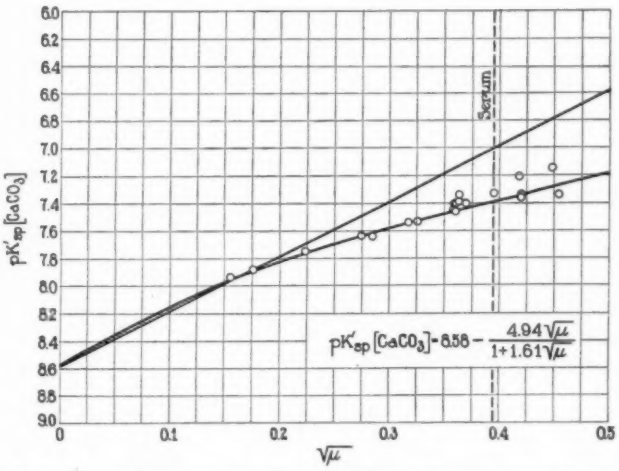

FIG. 1. The solubility of $CaCO_3$ in salt solutions of varying ionic strength saturated with $CaCO_3$ at 38°. Values of $pK'_{s.p.}$ $CaCO_3$ are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The curved line has the equation $pK'_{s.p.}$ $CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1+1.61\sqrt{\mu}}$.

Since it has been previously shown that $-\log \gamma_{CO_3^=} = 1.6\sqrt{\mu}$ as a first approximation up to $\mu = 0.160$, $\log \gamma_{Ca^{++}}$ can be calculated for any given ionic strength by means of the equation

$$-\log \gamma_{Ca^{++}} = \frac{4.94\sqrt{\mu}}{1 + 1.61\sqrt{\mu}} - 1.6\sqrt{\mu} \tag{64}$$

XUM

The manner in which the constants of equation (63) were obtained requires some explanation. The value 8.58 for $pK_{s.p.}$ $CaCO_3$ at $\mu = 0$ was obtained in four ways. (1) By graphic extrapolation of a smooth curve through the experimental points given in this paper; (2) by calculation of $pK_{s.p.}$ $CaCO_3$ at $\mu = 0$ from our experimental points at the two lowest ionic strength values where it may be assumed that the simple limiting relationship, $pK'_{s.p.} = pK_{s.p.} - 4\sqrt{\mu}$ holds; (3) by extrapolation of the curve obtained from a study of the solubility of $CaCO_3$ in solutions saturated with $Ca_3(PO_4)_2$ as well as $CaCO_3$ (these data are to be presented in Paper III of this series); (4) by calculation of $pK_{s.p.}$ $CaCO_3$ at $\mu = 0$ in the same manner as in method (2) from the data used in method (3). These methods of estimation led to the average value of 8.58 for $pK_{s.p.}$ $CaCO_3$ at $\mu = 0$. Since the lowest salt concentration in which the solubility of $CaCO_3$ was determined was $\mu = 0.024$, the error involved in extrapolation is considerable and makes the value 8.58 correspondingly questionable. Considering the possible errors involved however, it is felt that this value is probably within 0.1 of the true $pK_{s.p.}$ $CaCO_3$ at $\mu = 0.0$ and, as will be shown later, it agrees fairly well with previous determinations by other authors.

The manner in which the constants B and A were evaluated was as follows:

Equation (17) was converted to the form

$$pK_{s.p.}\,CaCO_3 - pK'_{s.p.}\,CaCO_3 = 2\,p\gamma_{CaCO_3} \tag{65}$$

But, by equation (21),

$$p\gamma_{CaCO_3} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}} \tag{66}$$

Therefore

$$\frac{pK_{s.p.}\,CaCO_3 - pK'_{s.p.}\,CaCO_3}{2} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}} \tag{67}$$

Rearranging and substituting 8.58 for $pK_{s.p.}CaCO_3$ equation (67) becomes

$$\frac{2}{8.58 - pK'_{s.p.}\,CaCO_3} = \frac{1 + A\sqrt{\mu}}{B\sqrt{\mu}} = \frac{1}{B}\left(\frac{1}{\sqrt{\mu}}\right) + \frac{A}{B} \tag{68}$$

By plotting values of $\left(\frac{2}{8.58 - pK'_{s.p.\ CaCO_3}}\right) = \frac{2}{\Delta\ pK'_{s.p.\ CaCO_3}}$ as ordinates against $\frac{1}{\sqrt{\mu}}$ as abscissæ, one obtains a straight line, the slope of which equals $\frac{1}{B}$ and the intercept of which on the y axis equals $\frac{A}{B}$. The values of A and B found in this way lead to the equation

$$pK'_{s.p.\ CaCO_3} = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.61\sqrt{\mu}} \tag{63}$$

It is appreciated that several of our points above $\sqrt{\mu} = 0.35$ lie above the line which represents this equation. Whether these deviations are significant and are not due to errors involved in manipulation and analysis is not known at the present time. The point of significance seems to be that the solubility of $CaCO_3$ in salt solutions of $NaHCO_3$ and NaCl increases with increasing salt concentration in a manner which is not inconsistent with the predictions of the Debye-Hückel theory.

TABLE V.

Activity Coefficients at 38° of $CaCO_3$, Ca^{++}, and $CO_3^{=}$ Calculated at Rounded Values of μ.

μ	$\gamma CaCO_3$	$\gamma CO_3^{=}$	γCa^{++}	γHCO_3^{-}	γCl^{-}	γH^{+}	γNa^{+}
0.00	1.0	1.0	1.0	1.0	1.0	1.0	1.0
0.05	0.39	0.44	0.35	0.77	0.84	0.88	0.84
0.10	0.31	0.31	0.31	0.69	0.81	0.84	0.80
0.15	0.26	0.24	0.28	0.64			
0.16*	0.25	0.23	0.27	0.63			

* Approximately the ionic strength of biological fluids.

Activity Coefficient of the Calcium Ion.

Since the activity coefficient of the carbonate ion in solutions of different ionic strength has been previously estimated, and since the activity coefficient of $CaCO_3$ has been determined, it is of some interest to estimate the activity coefficient of the calcium ion. This has been done by means of equation (64) and the results are tabulated in Table V for the activity coefficients at even ionic strengths.

For comparison we have given activity coefficients for the bicarbonate ion from our own data and for the H^+, Na^+, and Cl^- ions from Lewis and Randall's "Thermodynamics." It is particularly significant that the activity coefficients of the divalent ions $CO_3^=$ and Ca^{++} are much less than those of the monovalent ions HCO_3^- and Na^+. In the terminology of the electrolytic dissociation theory one would say that the divalent salts were less completely dissociated. The activity coefficients of Ca^{++} and $CO_3^=$ at $\mu = 0.160$ will be used to calculate the calcium activity in biological fluids.

It should be mentioned at this point that Bray (1925) in a classical investigation of HCO_3^- and $CO_3^=$ activity coefficients in salt solutions found a difference between the activity coefficient of the HCO_3^- ion as determined in systems composed of $NaHCO_3 - NaCO_3 - NaCl$ and those composed of $KHCO_3 - K_2CO_3 - KCl$. This suggests that one cannot use the same activity coefficient for the HCO_3^- ion in such biological systems as red blood cells which are rich in potassium, as in serum where sodium predominates.

Since in biological systems variations are encountered in p ($[Ca] \times [CO_3^=]$) other than those attributable to variations in ionic strength, certain conditions were varied in the salt solutions of the same ionic strength to determine what factors might there be significant.

The systems in which equilibrium with solid $CaCO_3$ was established will first be discussed, followed by the presentation of experiments in which equilibrium was apparently not established. The criterion of equilibrium has been regarded as the attainment of the value 7.30 to 7.40 for $pK'_{s.p.}$ $CaCO_3$ in solutions whose ionic strength was approximately $\mu = 0.150$. This was the value found in salt solutions containing no ions which might form with calcium other insoluble or slightly ionized compounds. This figure, within the limits studied, was independent of the $p\alpha_{H^+}$, the initial calcium concentration, and the duration of the saturation period beyond 16 hours. A shorter saturation period is undoubtedly adequate, although the minimum length of time necessary was not determined.

Conditions Leading to Equilibrium with $CaCO_3$ (Solid Phase: $CaCO_3$) (Liquid Phase: Solution of $NaCl + NaHCO_3$ with and without Calcium).

Salt solutions composed of all of the salts of serum except inorganic phosphate come rapidly into equilibrium with solid

TABLE VI.

Conditions Leading to Equilibrium with Solid $CaCO_3$. Saturation of Salt Solutions, Containing No Phosphate, with Solid $CaCO_3$.

Experiment No.	Time of equilibration.	$p\alpha_{H^+}$	$[CO_3]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	$pK'_{s.p.}$ $CaCO_3$	$\sqrt{\mu}$	Initially present. Calcium M × 10^3 / kg. H_2O	Initially present. Phosphate M × 10^3 / kg. H_2O
	hrs.								
1 (12)	44	7.51	31.84	0.160	0.33	7.28	0.387	0	0
2 (17)	44	7.39	27.30	0.103	0.46	7.33	0.374	7.76	0
3 (18′)	64	7.36	27.42	0.096	0.58	7.26	0.374	7.76	0

TABLE VII.

Conditions Leading to Equilibrium with Respect to $CaCO_3$. Saturation of Salt Solutions with $CaCO_3 + Ca_3(PO_4)_2$.

Experiment No.	Time of equilibration.	$p\alpha_{H^+}$	$[CO_3]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	$pK'_{s.p.}$ $CaCO_3$	$\sqrt{\mu}$	Initially present. Calcium M × 10^3 / kg. H_2O	Initially present. Phosphate M × 10^3 / kg. H_2O
1 (9)	20 hrs.	7.43	31.04	0.129	0.31	7.40	0.383	0	0
2 (14)	8 days.	7.51	31.35	0.157	0.26	7.39	0.387	0	0
3 (8)	20 hrs.	7.20	19.20	0.045	1.33	7.23	0.378	7.76	0
4 (16)	44 "	7.50	33.62	0.165	0.25	7.38	0.378	0	2.42
5 (24)	7 days.	7.57	33.67	0.195	0.23	7.37	0.380	0	2.42
6 (5)	20 hrs.	7.48	29.24	0.136	0.25	7.47	0.383	4.65	1.21
7 (20)	68 "	7.37	27.14	0.097	0.03	7.39	0.401	2.52	1.21

$CaCO_3$ whether there is no calcium initially in solution or whether there is an excess of calcium. Representative experiments illustrating this point are given in Table VI. These experiments show consistent values for $pK'_{s.p.}$ $CaCO_3$ which agree with those

obtained in the study of the solubility of $CaCO_3$ in solutions of varying ionic strength.

When we have the system (solid phase: $CaCO_3 + Ca_3(PO_4)_2$) (liquid phase: solution of NaCl + $NaHCO_3$, with and without

TABLE VIII.

Conditions Leading to Supersaturation with Respect to $CaCO_3$. Saturation in the Absence of a Solid Phase.

Experiment No.	Time of equilibration.	$p\alpha_{H^+}$	$[CO_3]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	$pK'_{s.p.}$ $CaCO_3$	$\sqrt{\mu}$	Initially present. Calcium M × 10^3 / kg. H_2O	Initially present. Phosphate M × 10^3 / kg. H_2O
	hrs.								
1 (18)	44	7.38	28.01	0.103	0.88	7.05	0.375	7.76	0.0
2	20	7.48	24.10	0.112	3.24	6.44	0.419	3.25	0.0
3 (19)	44	7.43	36.25	0.150	2.99	6.34	0.390	7.76	2.42
4 (22)	68	7.59	39.28	0.238	0.88	6.68	0.403	2.52	1.21
5 (27)	18	7.52	34.07	0.174	2.56	6.35	0.403	2.52	1.21

TABLE IX.

Conditions Leading to Supersaturation with Respect to $CaCO_3$. Saturation with $CaCO_3$ in the Presence of Phosphate in Solution.

Experiment No.	Time of equilibration.	$p\alpha_{H^+}$	$[CO_3]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	$pK'_{s.p.}$ $CaCO_3$	$\sqrt{\mu}$	Initially in solution. Calcium M × 10^3 / kg. H_2O	Initially in solution. Phosphate M × 10^3 / kg. H_2O
	hrs.								
1 (2)	20	7.54	33.69	0.181	1.23	6.65	0.382	0	1.21
2 (1)	20	7.45	31.39	0.136	1.26	6.77	0.387	4.65	1.21
3 (13)	42	7.41	29.93	0.118	1.42	6.78	0.380	7.76	1.21

calcium, with and without phosphate) equilibrium is established with respect to $CaCO_3$ whether or not calcium is initially present in the liquid phase. This is also true whether or not inorganic phosphate is initially present. Some experiments illustrating these results are given in Table VII.

Conditions Leading to Apparent Supersaturation. Absence of Solid Phase. (*Liquid Phase: Solution of* $NaHCO_3 + NaCl + CaCl_2$.)

In systems composed initially of only the liquid phase $NaHCO_3 - NaCl - CaCl_2$ and the gas phase CO_2, at a definite tension, equilibration for 44 hours did not result in complete equilibrium with the solid phase $CaCO_3$. This, as was anticipated, was a condition of supersaturation in which equilibration was not continued until equilibrium was established. The details of this experiment are given in Experiments 1 and 2 of Table VIII.

Absence of Solid Phase. (*Liquid Phase: Solution of* $NaHCO_3 + NaCl + CaCl_2 + \left\{\begin{matrix} Na_2HPO_4 \\ KH_2PO_4 \end{matrix}\right\}$.)

Since our ultimate goal was the study of calcium activity in biological fluids the previous experiments were repeated with the addition of a mixture of Na_2HPO_4 and KH_2PO_4 at physiological $p\alpha_{H^+}$ so that the total phosphate approximately equalled the concentration in serum. No solid phase was present. The length of saturation varied from 18 to 68 hours. The protocols of three of these experiments are also given in Table VIII (Nos. 3 to 5). In each instance the solution was apparently markedly supersaturated with respect to $CaCO_3$.

(*Solid Phase:* $CaCO_3$) (*Liquid Phase: Solution of* $NaHCO_3 + NaCl + CaCl_2 + \left\{\begin{matrix} Na_2HPO_4 \\ KH_2PO_4 \end{matrix}\right\}$).

If to the above aqueous phase, solid $CaCO_3$ is added, supersaturation with respect to $CaCO_3$ apparently still exists as is shown by the difference between $pK'_{s.p.}$ $CaCO_3$ values of 6.7 under these conditions as compared with 7.3 under conditions of equilibrium in systems without phosphates in solution (Table IX). It was further found that this condition of apparent supersaturation existed even though no calcium was initially in solution. Whether this condition is one of delayed equilibrium or complex ion formation is at present unknown.

Conditions Leading to Apparent Undersaturation. (Solid Phase: $Ca_3(PO_4)_2$) (Liquid Phase: Solution of $NaHCO_3$ + NaCl).

When the system composed initially of $NaHCO_3$ and NaCl was saturated with the solid phase $Ca_3(PO_4)_2$ at fixed CO_2 tensions, the liquid phase was unsaturated with respect to $CaCO_3$ even after 8 days (Table X). This was the case whether or not calcium or phosphate was initially present in the solution. The solubility product of $CaCO_3$ was not reached in this experiment.

TABLE X.

Conditions Leading to Undersaturation. Saturation of Salt Solutions with $Ca_3(PO_4)_2$.

Experiment No.	Time of equilibration.	p_{H^+}	$[CO_2]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	pK′ s.p. $CaCO_3$	$\sqrt{\mu}$	Initially in solution. Calcium M × 10^3 / kg. H_2O	Initially in solution. Phosphate M × 10^3 / kg. H_2O
1 (7)	20 hrs.	7.37	27.14	0.097	0.05	8.31	0.381	0	0
2 (10)	8 days.	7.47	28.53	0.130	0.03	8.41	0.385	0	0
3 (6)	20 hrs.	7.18	18.17	0.041	0.59	7.62	0.376	7.76	0
4 (4)	20 "	7.38	25.66	0.094	0.04	8.43	0.379	0	1.21
5 (3)	20 "	7.32	25.05	0.079	0.04	8.50	0.384	4.65	1.21

Discussion of Experiments upon Solubility of $CaCO_3$ in Salt Solutions.

It is a thermodynamic necessity that in all solutions in equilibrium with solid $CaCO_3$ the activity and the activity solubility product of $CaCO_3$ are the same. For 38° we have found this latter value (designated as $K_{s.p.}$) to be $1 \times 10^{-8.58}$. Since the activity coefficients of the calcium and carbonate ions decrease from unity as the ionic strength of the solutions increases from zero, the analytically determined values of Ca^{++} and $CO_3^=$ will become progressively greater as will the product $[Ca^{++}] \times [CO_3^=]$.

In the foregoing experiments, it has been shown that salt solutions containing no phosphate may be brought into equilibrium with solid calcium carbonate and that the analytically determined product $[Ca^{++}] \times [CO_3^=]$ is a constant which varies with

the ionic strength of the solution in a manner which is in harmony with the Debye and Hückel theory of the behavior of strong electrolytes.

From our data we have calculated activity coefficients for Ca^{++} and $CO_3^{=}$ ions in simple salt solutions of varying ionic strength. Is one justified in applying these activity coefficients to all solutions the ionic strengths of which are known, and which have been equilibrated with solid $CaCO_3$ until no further change in the analytical product $[Ca^{++}] \times [CO_3^{=}]$ occurs? Perhaps not, because as Holt, La Mer, and Chown (1925, *b*) pointed out, inhibiting influences may delay the attainment of equilibrium for a long period of time. However, since this method of attack seems at present the most fruitful, we have used it in subsequent sections on serum.

It is further appreciated that there are specific ion effects of considerable magnitude in solutions composed of salts other than $NaHCO_3$ and NaCl which will markedly affect the activity coefficients of the calcium and carbonate ions. In the absence of information upon the magnitude of these specific influences, however, we have used the activity coefficients found in our experiments on salt solutions.

A point of interest and importance for the interpretation of our later work on biological fluids arises from our experiments upon $CaCO_3$ solubility in salt solutions containing inorganic phosphate in solution. It will be recalled that the analytical product $[Ca] \times [CO_3^{=}]$ was markedly greater than one found in entirely comparable experiments but in which no phosphate was initially present in solution. Furthermore, this product was practically the same whether one started with calcium in solution or not. Was the activity product $\alpha_{Ca^{++}} \times \alpha_{CO_3^{=}}$ greater in these solutions than in others without phosphate? It is highly improbable that $CaCO_3$ had been formed but not precipitated in view of the fact that an excess of solid salt was constantly present. Nor does it seem possible that the presence of the small amount of phosphate was in itself sufficient to alter the activity coefficient of the Ca^{++} and $CO_3^{=}$ in the presence of phosphate when both solid phases, $CaCO_3$ and $Ca_3(PO_4)_2$, were present.

Since solid $Ca_3(PO_4)_2$ was absent in these experiments it is possible that the calcium and the phosphate were combined

but not precipitated. If this is true, as will be explained later, we are justified in applying our activity coefficients for Ca^{++} and $CO_3^{=}$ found for salt solutions containing no phosphate calculating then the calcium ion concentration, subtracting this from the total calcium analytically determined, and calling this value unionized calcium. This has been our procedure in the work now to be described and to which the Debye and Hückel theory did not seem applicable without some such hypothesis.

An alternative explanation of these results is that the reaction between Ca^{++} and $CO_3^{=}$ was inhibited by the presence of the phosphate and a condition of delayed equilibrium resulted. If this were the true explanation, the thermodynamic method of treatment would be inapplicable to this experiment and the comparable condition found in serum. Realizing this, we must emphasize that our subsequent thermodynamic treatment serves as a method of attack which is applicable only if equilibrium exists. Whether it does or not has not as yet been rigidly determined.

Solubility of $CaCO_3$ in Solutions of Sodium Citrate.

Having determined the solubility of $CaCO_3$ in solutions of $NaHCO_3$ and NaCl, and having studied some of the factors which seem to prevent the attainment of equilibrium with respect to $CaCO_3$, it was decided to study the effect of sodium citrate upon the solubility of $CaCO_3$. Complex calcium citrate ions are supposed to exist but we were unable to obtain accurate information regarding their exact nature. Since the citrate ion is a trivalent ion it was at first thought that its action in dissolving $CaCO_3$ might be explicable merely upon the basis of an increased ionic strength of the solution. This proved to be an inadequate explanation for several reasons. First, because the solubility product was not constant at varying $p\alpha_{H^+}$ although the ionic strength was constant; second, because when values of $pK'_{a.p.}$ $CaCO_3$ chosen at constant $p\alpha_{H^+}$ were plotted against the $\sqrt{\mu}$ the curve did not conform to that predicted by the Debye and Hückel theory. To explain our results one would have to assume a large specific ion effect or the existence of a slightly dissociated calcium citrate compound. Our calculations have been made on the basis of the latter hypothesis.

In the simplest case, the liquid phase was initially composed only of a solution of sodium citrate, $Na_3C_6H_5O_7$. In some cases $NaHCO_3$ was also initially present. These solutions were equilibrated with solid $CaCO_3$ at definite CO_2 tensions which, however, changed during the course of the saturation. The technique and analytical procedures were those which have been already described. The results of our experiments are given in Table XI. At equilibrium the liquid phase contained the following: $Na_3C_6H_5O_7$, $NaHCO_3$, $Ca_3(C_6H_5O_7)_2$, $Ca(HCO_3)_2$, H_2CO_3. The reactions concerned with this change in composition are

$$\underset{\text{Solid.}}{CaCO_3} \rightleftarrows \underset{\text{Solution.}}{CaCO_3} + H_2CO_3 = Ca(HCO_3)_2$$

$$2Na_3C_6H_5O_7 + 3Ca(HCO_3)_2 = Ca_3(C_6H_5O_7)_2 + 6NaHCO_3$$

The $p\alpha_{H^+}$ was sufficiently high so that forms of citrate other than the tertiary salt can be neglected.

The ionic strength of the solution at equilibrium being known and the $[CO_3^{=}]$ concentration having been calculated, it is possible to calculate the $[Ca^{++}]$ concentration which one would expect to find in a NaCl solution of the same ionic strength. It is appreciated that this is only an approximation because the specific ion effect in the case of the citrate ion is probably very different from that of the chloride ion. The total calcium content having been found by analysis, it is possible on the basis of the assumptions made with reference to $\gamma_{Ca^{++}}$, to estimate the amount of non-ionized calcium (Column 8, Table XI).

Since $Ca_3(C_6H_5O_7)_2$ would be expected to ionize according to the equation

$$Ca_3(C_6H_5O_7)_2 \rightleftarrows 3Ca^{++} + 2C_6H_5O_7^{\equiv}$$

the following equation might be expected to prevail at equilibrium:

$$\frac{[Ca^{++}]^3 \times [C_6H_5O_7^{\equiv}]^2}{[Ca_3(C_6H_5O_7)_2]} = K'_{Ca_3(C_6H_5O_7)_2} \tag{69}$$

or

$$3pCa^{++} + 2p(C_6H_5O_7^{\equiv}) - pCa_3(C_6H_5O_7)_2 = pK'_{Ca_3(C_6H_5O_7)_2} \tag{70}$$

We have attempted to calculate $pK'_{Ca_3(C_6H_5O_7)_2}$ in the experiments reported with the result shown in Column 9. There seems to be a correlation between the $p\alpha_{H^+}$ and the value $pK'_{Ca_3(C_6H_5O_7)_2}$ as Fig. 2 indicates. Whether this means that at higher $p\alpha_{H^+}$ values the salt is of different composition from that at the lower values is unknown at present. A slow rearrangement from the salt $CaH(C_6H_5O_7)$ to the salt $Ca_3(C_6H_5O_7)_2$ has been described

TABLE XI.

Solubility of $CaCO_3$ in Solutions of Sodium Citrate.

Experiment No. (1)	$\sqrt{\mu}$* (2)	$p\alpha_{H^+}$ (g) (3)	$[CO_3]$ M × 10^3 / kg. H_2O (4)	$[CO_3^{=}]$ M × 10^3 / kg. H_2O (5)	Calculated $[Ca^{++}]$ M × 10^3 / kg. H_2O (6)	Total [Ca] M × 10^3 / kg. H_2O (7)	[CaX] M × 10^3 / kg. H_2O (8)	$pK'_{Ca_3(C_6H_5O_7)_2}$ (9)	Initial concentration of $Na_3C_6H_5O_7$ M × 10^3 / kg. H_2O (10)
1	0.549	7.058	48.90	0.123	0.59	17.22	16.63	10.52	40
2	0.540	7.33	40.43	0.200	0.36	14.13	13.77	11.00	40
3	0.561	7.30	54.80	0.263	0.29	25.92	25.63	11.83	40
4	0.478	6.90	38.53	0.055	1.10	16.76	15.66	10.64	20
5	0.475	7.52	31.86	0.204	0.29	15.39	15.10	12.32	20
6	0.475	6.81	37.15	0.041	1.45	15.73	14.28	10.16	20
7	0.470	6.92	30.80	0.044	1.32	13.43	12.11	10.09	20
8	0.467	7.29	27.14	0.098	0.58	12.78	12.20	10.19	20
9	0.465	7.62	22.76	0.182	0.31	11.08	10.77	11.87	20
10	0.465	7.82	22.55	0.288	0.20	11.08	10.88	12.47	20
11	0.425	7.38	22.10	0.089	0.53	10.53	10.00	12.32	10
12	0.426	7.04	24.05	0.042	1.12	10.79	9.67	10.26	10
13	0.425	7.50	21.83	0.117	0.40	10.53	10.13	12.71	10
14	0.392	6.77	15.93	0.012	3.20	6.56	3.36	9.64	5
15	0.384	7.59	8.44	0.050	0.78	4.07	3.29	11.47	5
16	0.388	6.76	31.85	0.024	1.66	5.40	3.80	10.66	5
17	0.380	7.61	21.87	0.138	0.28	3.02	2.74	12.64	5

* Calculated from final composition of solutions.

(Pannozani, 1910), and it may be that some such phenomenon plays a rôle here.

The important points illustrated by the results of these experiments are:

1. The fact that the product $[Ca] \times [CO_3^{=}]$ is not a constant at constant ionic strength but varies with the $p\alpha_{H^+}$ of the solution and with the concentration of citrate present.

2. That this cannot be explained on the basis of supersaturation or by the Debye-Hückel theory, without the assumption of an unusually large specific ion effect.

3. That it *does* seem to be explicable on the hypothesis of the formation of a slightly ionized calcium compound.

4. This calcium citrate compound does not seem to be a simple substance such as $Ca_3(C_6H_5O_7)_2$.

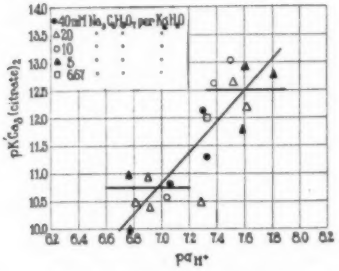

FIG. 2. The apparent variation of $pK'Ca_3(C_6H_5O_7)_2$ with $p\alpha_{H^+}$. The solutions containing different total citrate concentrations are designated by different symbols.

● = 40 mM sodium citrate per kilo of H_2O.
△ = 20 " " " " " " " "
○ = 10 " " " " " " " "
□ = 6.67 " " " " " " " "
▲ = 5 " " " " " " " "

Precipitation of Calcium from a Citrate Solution in Equilibrium with Solid $CaCO_3$ by the Addition of Solid $Ca_3(PO_4)_2$.

In a later section it will be shown that serum, when equilibrated with $CaCO_3$ does not alter in its calcium concentration, but when it is equilibrated with $Ca_3(PO_4)_2$ calcium is precipitated. In order to determine whether solutions of citrate, in equilibrium with solid $CaCO_3$, showed the same behavior, the following experiment was performed. 40 cc. of a solution of sodium citrate (6.67 mM per liter) were saturated 20 hours at 38° with solid $CaCO_3$ at a CO_2 tension of approximately 25 mm. At the close of the saturation period the solution was separated from the solid

$CaCO_3$ and analyzed for CO_2, calcium, and $p\alpha_{H^+}$. It was then saturated for 20 hours at 38° with $Ca_3(PO_4)_2$ and again analyzed. The results of this experiment are given in Table XII. That precipitation of calcium does occur is strikingly shown, thereby illustrating a property of citrate solutions which resembles that of serum.

Solubility of $CaCO_3$ in Serum.

The results of our study of the solubility of calcium salts in serum will now be presented. In this section only the experiments related to $CaCO_3$ solubility will be considered; in later sections the solubility of $Ca_3(PO_4)_2$ will be discussed.

TABLE XII.

Effect of Solid $Ca_3(PO_4)_2$ upon a Citrate Solution in Equilibrium with $CaCO_3$.

Initial concentration of $Na_3C_6H_5O_7$ = 0.00667 M. Saturation 20 hours with solid $CaCO_3$ at 38°.

μ	$\sqrt{\mu}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pK'_{a.p.}$ $CaCO_3$ calculated.	$pCO_3^=$	pCa^{++}	$[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$Ca_3(C_6H_5O_7)_2$ $\frac{M \times 10^3}{kg.\ H_2O}$	H_2O (calculated) $\frac{gm.}{cc.}$
0.057	0.240	5.84	7.33	12.65	0.0273	7.72	4.564	3.16	0.692	5.15	0.994
Same solution saturated 20 hours with solid $Ca_3(PO_4)_2$ at 38°.											
0.057	0.240	3.78	7.03	8.63	0.00872		5.06				

If one analyzes blood serum as drawn from the body without permitting a change in the gases of the blood to occur, one may obtain values of the $p\alpha_{H^+}$ and CO_2 content from which $[CO_3^=]$ ion concentration may be calculated.

The $[CO_3^=]$ ion concentration has been calculated from the $p\alpha_{H^+}$ and $[CO_2]$ content on the assumption that the activity coefficients of the $[HCO_3^-]$ ion and the $[CO_3^=]$ ion are identical in serum with the values which were found for them in a solution of $NaHCO_3$ and NaCl the ionic strength of which was 0.160. That this assumption is probably nearly correct for the HCO_3^- ion is indicated by the fact that the value for the activity coefficient of

the HCO_3^- ion was found to be approximately the same in serum as in a salt solution of $\mu = 0.160$. We have no direct evidence that the activity coefficient of the $CO_3^=$ ion is identical in serum and in a salt solution of the same ionic strength.

The $[CO_3^=]$ ion concentration when multiplied by the total Ca concentration gives the product $[Ca] \times [CO_3^=]$. The corresponding product in simple salt solutions containing no slightly ionized calcium and in equilibrium with solid $CaCO_3$, is the solubility product constant of calcium carbonate in this solution.

Analyses of serum show that the product $[Ca] \times [CO_3^=]$ far

TABLE XIII.

Determination of the p ($[Ca] \times [CO_3^=]$) in Human Serum.

Experiment No.	p_{H^+}	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pCO_3^=$	pCa	$p([Ca] \times [CO_3^=])$	$p([Ca] \times [CO_3^=])$ calculated to $p_{H^+} = 7.40$.	Remarks.
1	7.47	30.40	0.138	3.28	3.86	2.48	6.34	6.40	Normal human.
2	7.42	31.20	0.126	2.95	3.90	2.53	6.43	6.45	" "
3	7.44	31.05	0.132	2.82	3.88	2.55	6.43	6.47	" "
4	7.42	33.33	0.135	2.80	3.87	2.55	6.42	6.44	" "
5	7.33	31.03	0.101	2.94	4.00	2.53	6.53	6.47	" "
6	7.49	27.43	0.131	2.58	3.88	2.59	6.47	6.55	Nephrosis. Low plasma proteins.
7	7.34	32.92	0.110	2.29	3.96	2.64	6.60	6.55	" "

Salt solution: $\mu = 0.160$. $pK'_{s.p.}\ CaCO_3 = 7.40$.

exceeds that found for salt solutions of similar ionic strength. The ionic strength of serum has been estimated from the analysis of the constituents of serum to be 0.167 (Van Slyke and collaborators, 1925) where proteins are assumed to behave as univalent anions. In Table XIII have been collected the results of analyses of normal and pathological serum as drawn. The $pK'_{s.p.}$ of $CaCO_3$ for a salt solution of similar ionic strength has been given for comparison.

The great discrepancy in the values for $p([Ca] \times [CO_3^=])$ between salt solutions not containing phosphate and in equi-

librium with solid $CaCO_3$, on the one hand, and serum *in vivo*, on the other hand, gives rise to a question of great interest and importance; namely, the significance of this product in serum as drawn, and the factors which are responsible for it. Several possibilities suggest themselves as an explanation for the existence of calcium in the amounts in which it occurs in serum.

(1) Serum in the body may not have direct access to the bone in the manner of a solution saturated with a solid salt. Hence not being in equilibrium with bone, serum *in vivo* may be supersaturated or undersaturated with respect to the calcium salts. (2) One or both of these salts may be present in finely divided form in suspension. (3) Whether in equilibrium with bone or

TABLE XIV.

Effect of Saturating Normal Horse Serum with Solid $CaCO_3$ for Varying Lengths of Time at 38°.

Experiment No.	Length of saturation period.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca^{++}]$ calculated $\frac{M \times 10^3}{kg.\ H_2O}$	$[CaX]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$	$p([Ca] \times [CO_3^=])$ at $p\alpha_{H^+} = 7.40$.
1	0	7.47	31.07	29.68	0.141	3.37	0.28	3.09	6.32	6.38
2	5 min.	7.44	31.35	29.74	0.133	3.36	0.30	3.06	6.35	6.38
3	1 hr.	7.43	31.39	29.77	0.130	3.36	0.31	3.05	6.36	6.38
4	22 "	7.17	19.07	17.44	0.042	3.46	0.96	2.50	6.84	6.63

not, calcium may be present in serum in an amount governed merely by the specific ion effect of the proteins or other substances to be found in serum, not present in our simple salt solutions. (4) Calcium in serum may be present as part of a complex ion or molecule of a substance, which is but slightly dissociated. Evidence will be offered in the following sections in support of our belief in the last named hypothesis.

To test the first possibility, horse serum was shaken at physiological CO_2 tensions in contact with solid $CaCO_3$ for varying lengths of time. At constant $p\alpha_{H^+}$ no change occurred in the total calcium up to 22 hours. The value of $p([Ca] \times [CO_3^=])$ was greater at the end of the saturation period but the $p\alpha_{H^+}$ was

lower, which, as later experiments show, practically accounts for the increase in this product (Table XIV).

This might suggest that the serum *in vivo* was already in equilibrium with solid $CaCO_3$ in the body. If this were indeed true, it seemed that equilibration of the serum with solid $CaCO_3$ after depletion of the calcium content of serum should result in the solution of $CaCO_3$, or augmentation of the calcium content should result in precipitation of calcium until the original product [Ca] $\times$ [$CO_3^{=}$] was satisfied. This was not simply realized, however, as the following experiments show.

TABLE XV.

Experiment Illustrating Failure of Serum to Dissolve $CaCO_3$ after Partial Precipitation of Calcium Originally Present by $Na_2C_2O_4$.

Original calcium concentration = 3.01 mM per kilo of H_2O.
After precipitation with $Na_2C_2O_4$, Ca = 0.64 mM per kilo of H_2O.
Saturated with solid $CaCO_3$ at 38° for 24 hours.

Sample.	$p\alpha_{H^+}$	$\frac{[CO_2]}{M \times 10^3 / kg.\ H_2O}$	$\frac{[CO_3^{=}]}{M \times 10^3 / kg.\ H_2O}$	$\frac{[Ca]}{M \times 10^3 / kg.\ H_2O}$	$pCO_3^{=}$	pCa	$p([Ca] \times [CO_3^{=}])$
1	7.02	36.52	0.0548	0.596	4.26	3.22	7.48
2	7.18	33.53	0.0751	0.511	4.12	3.29	7.41
3	7.34	29.36	0.0978	0.404	4.01	3.39	7.40

Solubility of $CaCO_3$ in Serum after Partial Precipitation of the Calcium as Calcium Oxalate.

The calcium of serum was partially precipitated as calcium oxalate by the addition of a weighed amount of sodium oxalate. After allowing the serum to stand, the calcium oxalate was removed by centrifugation and the serum was equilibrated for 24 hours with solid $CaCO_3$. At the end of the saturation period the serum was analyzed for its $p\alpha_{H^+}$ and CO_2 and calcium content. The results of this experiment are given in Table XV. They show that $CaCO_3$ did not dissolve again after the calcium content had been depleted by precipitation as calcium oxalate.

That the serum had not lost its power to hold calcium in solution, however, is shown by Table XV.

After precipitation of the calcium as calcium oxalate, $CaCl_2$

was added to the serum in a quantity comparable to its original calcium content. This serum was then equilibrated with solid calcium carbonate and analyzed after 20 hours. The results of this experiment, given in Table XVI, show that the serum had not lost its power to hold calcium in solution.

TABLE XVI.

Experiment Illustrating Ability of Serum to Hold $CaCl_2$ in Solution after Precipitation of the Original Calcium with $Na_2C_2O_4$.

Original calcium concentration = 3.28 mM per kilo of H_2O.
After addition of $Na_2C_2O_4$, Ca = 1.11 " " " " "
" " " $CaCl_2$, Ca = 3.25 " " " " "
Saturated with solid $CaCO_3$ at 38° for 20 hours.

Experiment No.	$p\alpha_{H^+}$	$\frac{[CO_2]}{M \times 10^3 / kg.\ H_2O}$	$\frac{[CO_3^=]}{M \times 10^3 / kg.\ H_2O}$	$\frac{[Ca]}{M \times 10^3 / kg.\ H_2O}$	$pCO_3^=$	pCa	$p([Ca] \times [CO_3^=])$
1	6.99	29.48	0.041	2.94	4.39	2.53	6.92
2	6.97	27.73	0.0366	2.995	4.44	2.52	6.96
3	6.92	28.40	0.0336	2.93	4.48	2.53	7.01

TABLE XVII.

Effect of Saturating Horse Serum with $CaCO_3$ after Increasing Original Calcium Concentration by Addition of Ca $(HCO_3)_2$.

Experiment No.	Length of saturation period.	$p\alpha_{H^+}$	$\frac{M \times 10^3}{kg.\ H_2O}$ $[CO_2]$	$\frac{M \times 10^3}{kg.\ H_2O}$ $[CO_3^=]$	$\frac{M \times 10^3}{kg.\ H_2O}$ [Ca]	$p([Ca] \times [CO_3^=])$	$p([Ca] \times [CO_3^=])$ at $p\alpha_{H^+} = 7.40$
1	0	7.50	35.71	0.175	7.09	5.91	5.99
2	5 min.	7.40	37.03	0.143	7.02	6.00	6.00
3	1 hr.	7.59	35.24	0.214	7.02	5.82	5.97
4	3 "	7.54	35.90	0.193	7.00	5.87	5.98
5	22 "	7.39	34.58	0.131	7.09	6.03	6.02

Solubility of $CaCO_3$ in Serum after Augmentation of the Original Calcium Concentration.

To serum was added $Ca(HCO_3)_2$ in an amount sufficient to double the original calcium content of serum. This serum was then equilibrated with solid $CaCO_3$ and analyzed after varying

lengths of time. Apparently, none of the added calcium was precipitated, as shown by the results of this experiment (Table XVII). Furthermore, the log product, $p([Ca] \times [CO_3^{=}])$ was markedly constant throughout the 22 hours of saturation.

Solubility of $CaCO_3$ in Serum after Removal of Phosphates and Augmentation of the Calcium.

Having found in later experiments that equilibration of serum with augmented calcium at $p\alpha_{H^+}$ above 8.0, in the presence of

TABLE XVIII.

Experiment upon Solubility of $CaCO_3$ in Horse Serum after Increasing Calcium Concentration and Saturating Serum with Solid $CaCO_3$ and $Ca_3(PO_4)_2$.

Experiment No.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^{=}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^{=}])$	Remarks.
1	7.40*			3.48		Initial condition.
2				6.55		After addition of $Ca(HCO_3)_2$.
3	>8.5			2.51		" saturation with $CaCO_3$ + $Ca(PO_4)_2$.
4	>8.5			6.20		After addition of $CaCl_2$.
5	>8.5			1.95		" saturation with $CaCO_3$, alone.
6	7.15	13.80	0.0288	2.56	7.13	After equilibration with $CaCO_3$, alone, at 25 mm. CO_2 tension.

* Assumed.

solid $CaCO_3$ and $Ca_3(PO_4)_2$, reduces the amount of inorganic phosphate in solution, and believing that the presence of the inorganic phosphate of serum may have prevented the precipitation of $CaCO_3$ in the previous experiment, serum was treated in this manner until no determinable amount of inorganic phosphate remained in solution; *i.e.*, the concentration was less than 0.01 mM per kilo of H_2O. The calcium concentration at this time was 2.51 mM. Calcium chloride was then added until the calcium concentration was 6.20 mM, and this serum was saturated with

solid $CaCO_3$ at $p\alpha_{H^+} = 8.5$. After 18 hours the calcium concentration was 1.95 mM, showing that 4 mM of $CaCO_3$ had been precipitated by saturation with solid $CaCO_3$. It should be noted, however, that phosphates were now absent from the serum. Continued saturation of the serum with $CaCO_3$ at 25 mM CO_2 tension at a $p\alpha_{H^+}$ of 7.15 resulted in the solution of 0.6 mM of calcium, the amount which one would expect to dissolve in a salt solution of serum ionic strength and $CO_3^{=}$ concentration, as one passed from $p\alpha_{H^+}$ 8.5 to 7.15. These results are shown in Table XVIII. The total amount of calcium in solution finally, 2.56 mM, was still about 1 mM more than one can account for simply by the solubility of $CaCO_3$ and the effect of the proteins, and this in the absence of phosphates. This experiment seems to indicate, therefore, that in the absence of phosphates, calcium is precipitated by saturation with $CaCO_3$ from serum even though it has been greatly increased in concentration.

Taken in conjunction with the previous experiment, it would appear that to the presence of phosphates in the serum may be assigned, in part at least, the rôle of delaying or preventing the precipitation of calcium after augmentation of the calcium. That the presence of the phosphates is not necessary for the maintenance of the calcium level above that accounted for by the salts and the proteins is also shown. It is recognized, however, that with the precipitation of the phosphates, the precipitation of some other factor which is involved in the maintenance of the normal calcium level is not precluded.

Effect of Varying $p\alpha_{H^+}$ upon the Solubility of $CaCO_3$.

The $p\alpha_{H^+}$ of the serum was varied from approximately 6.0 to 8.0 by varying the CO_2 tension, and the serum rotated in contact with solid $CaCO_3$. The results of one such experiment are given in Table XIX and graphically presented in Fig. 3. It will be seen that only at the highest $p\alpha_{H^+}$ was there a significant drop in calcium. This was accompanied by a drop in phosphate. Calculating the precipitated salt as $Ca_3(PO_4)_2$ the decrease in phosphate corresponded to a decrease in calcium of 0.43 mM as compared with an observed fall of 0.58 mM. It is of more importance, however, that the total calcium concentration did not

TABLE XIX.

Solubility of $CaCO_3$ in Horse Serum Saturated with Solid $CaCO_3$ at 38° and Varying $p\alpha_{H^+}$ Values.

Length of saturation, 16 hours.

Experiment No.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Calculated $[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CaX]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$
1	6.30	58.03	0.011	3.23	0.76	3.63		7.45
2	6.91	39.61	0.045	3.23	0.75	0.89	2.34	6.84
3	7.51	31.60	0.158	3.21	0.74	0.25	2.96	6.29
4	7.97	28.36	0.397	3.14	0.73	0.10	3.04	5.90
5	8.46	24.37	1.084	2.65	0.48	0.036	2.61	5.54

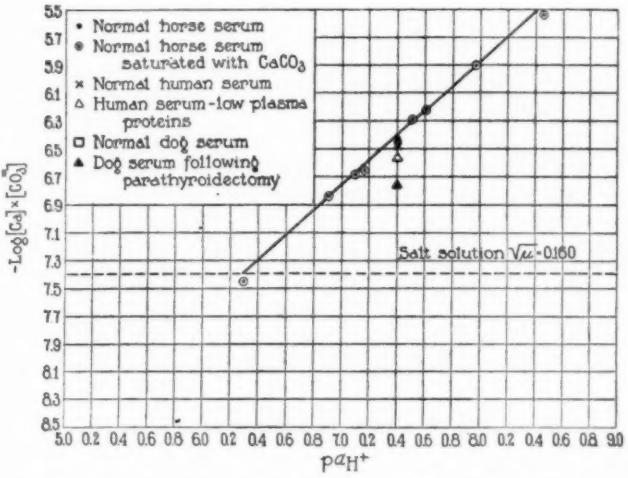

FIG. 3. The negative log product of $[Ca] \times [CO_3^=]$ in serum under varying conditions plotted against $p\alpha_{H^+}$.

change from $p\alpha_{H^+} = 7.5$ to $p\alpha_{H^+} = 6.3$. Because of the change which occurs in $CO_3^=$ ion concentration with changing $p\alpha_{H^+}$, the negative log product $pK'_{s.p.}$ $CaCO_3$ varies progressively with

the $p\alpha_{H^+}$. At $p\alpha_{H^+}$ 6.3 to 6.4, $p([Ca] \times [CO_3^{=}])$ equals that obtained in salt solutions of the same ionic strength.

Assuming that each of these solutions was in equilibrium with solid $CaCO_3$ it is possible to calculate $[Ca^{++}]$ and [CaX] at each $p\alpha_{H^+}$. The results of such a calculation are given in Table XIX.

These experiments have been repeated several times with the same result. They show the variation of CaX with changing $p\alpha_{H^+}$ and suggest the hypothesis that up to a certain point CaX exists in greater amount at more alkaline reactions. Were it a calcium proteinate compound which were concerned, this phenomenon would be understandable, because it is well established that serum proteins bind more base at higher $p\alpha_{H^+}$ values.

It may be felt that in a solution containing such a preponderance of sodium salts, one would not expect a significant amount of calcium proteinate to be formed. However, as the citrate experiments presented in the previous section indicate, when a slightly ionized compound is formed, one may reasonably look for an increased amount of calcium.

Influence of Serum Concentration upon $CaCO_3$ Solubility.

To determine whether the [CaX] concentration varied proportionally to the amount of serum in the solution equilibrated with $CaCO_3$, horse serum was diluted with known proportions of a salt solution of the same inorganic salts of serum and of the same ionic strength. These solutions were equilibrated with solid $CaCO_3$ at definite CO_2 tensions. The results of such an experiment are given in Table XX and the amount of CaX has been calculated for each concentration of serum. This is approximately proportional to the concentration of serum in the solution equilibrated although the fact that the $p\alpha_{H^+}$ values are not identical makes it impossible to compare strictly the results with each other.

Influence of the Proteins of Serum upon $CaCO_3$ Solubility.

Serum was dialyzed in collodion bags until chloride-free, then NaCl and $NaHCO_3$ were added until a solution of serum ionic strength was obtained. Calcium was also added in the form of $CaCl_2$ so that its initial concentration was 5 mM. This solution

was equilibrated with solid $CaCO_3$ at varying $p\alpha_{H^+}$, and analyzed for total Ca, CO_2, and $p\alpha_{H^+}$. The results of these experiments are given in Table XXI. The amount of unionized calcium per

TABLE XX.

Solubility of $CaCO_3$ in Serum Diluted with Varying Amounts of Salt Solution of Same Ionic Strength.

Composition of Solution.

	per cent		per cent
Experiment 1.	Serum, 100	Salt solution,	0
" 2.	" 60	" "	40
" 3.	" 30	" "	70
" 4.	" 10	" "	90

Each equilibrated 20 hours with solid $CaCO_3$.

Experiment No.	$p\alpha_{H^+}$ (e)	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pCO_3^=$	pCa	$[CaX]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$
1	6.97	20.93	0.0276	3.15	4.56	2.50	1.82	7.06
2	7.08	22.12	0.0387	1.84	4.41	2.74	0.94	7.15
3	7.14	26.73	0.0545	0.95	4.26	3.02	0.35	7.28
4	7.30	28.68	0.0863	0.58	4.06	3.24	0	7.30

TABLE XXI.

Solubility of $CaCO_3$ in Dialyzed Horse Serum, $\mu = 0.150$. Total Protein = 57 Gm. per Kilo of H_2O.

Experiment No.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$	$[CaX]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$\frac{[CaX]}{[P]}$
1	7.67 c	32.60	0.2380	1.075	6.59	0.93	0.0163
2	7.48 c	30.38	0.1415	1.075	6.82	0.83	0.0145

gm. of protein was found to be 0.0163 mM at $p\alpha_{H^+} = 7.67$ and 0.0145 mM at $p\alpha_{H^+} = 7.48$. Extrapolated to $p\alpha_{H^+} = 7.4$, the value for the amount of calcium bound per gm. of protein is 0.0137 mM.

The amount of calcium bound by serum proteins may be evaluated also in the following way. By plotting the amount of calcium found in serum and transudates of serum containing amounts of protein varying from 0.0 to 80 gm. per kilo of water against the protein concentration, one obtains the curve given in Fig. 4. The data from which this figure is made are taken partly from data published by Salvesen and Linder, and partly from data obtained in connection with this investigation. These data may be considered to be those obtaining in solutions at $p\alpha_{H^+} = 7.4$ and of a constant ionic strength, approximately 0.165. The equation for the line relating the calcium to the protein concentration is

$$[Ca] = 0.014\,[P] - 1.4 \tag{71}$$

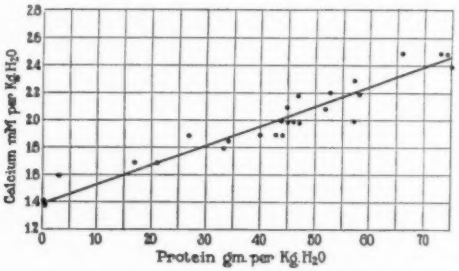

FIG. 4. The total calcium of serum and transudates plotted against the protein concentration. The straight line has the equation [Ca] = 0.014 P − 1.4.

where [Ca] = total calcium concentration in mM per kilo of H_2O, and [P] = gm. of protein per kilo of H_2O. These data taken from human serum and transudates as drawn from the body indicate, therefore, that the calcium bound per gm. of protein = 0.014 mM at $p\alpha_{H^+} = 7.4$. This compares well with the value of 0.0137 mM estimated for horse serum proteins.

Although our data are admittedly scanty, it is of interest to use them to derive a relationship between the calcium bound by protein, the protein concentration, and the $p\alpha_{H^+}$. Such an approximate expression fitting our results obtained upon solutions of dialyzed serum protein is

(72) $[CaP] = 0.01\ [P]\ (p\alpha_{H^+} - 6.0)$ where

$[CaP]$ = the calcium bound by protein in mM Ca per kilo of H_2O

$[P]$ = the protein concentration, in gm. per kilo of H_2O

The above relationship is purely empirical and holds only for the conditions in which the salt concentrations are those found in serum.

The question may be raised as to whether the increase in the ionic strength of the serum due to the proteins may not be sufficient to account for the increase in calcium due to their presence. This we do not believe to be the case for reasons given previously and in the following. Assuming that the increase *is* due to an increased ionic strength of the solution, and that the following formula holds

$$\Delta p\gamma_{Ca^{++}} = 0.5\ z^2(\sqrt{\mu_1} - \sqrt{\mu_2})$$

In a salt solution of $\mu = 0.170$ without protein, the concentration of calcium at $p\alpha_{H^+} = 7.4$ is about 0.4 mM. In serum under similar conditions, it is about 1.4 mM. Now $\frac{0.4}{1.4}$ may be considered to be equal to the change in the activity coefficient in passing from a solution without protein to serum. For simplicity we shall calculate the increase in μ as if we passed from 0.0 to μ ionic strength. Therefore

$$\Delta p\gamma_{Ca^{++}} = -\log \frac{0.4}{1.4} = 0.5 \times 2 \times 2\sqrt{\mu} = 2\sqrt{\mu}$$

Solving for μ, one finds it equal to 0.073. If the ionic strength of serum were 0.073 more than the value of 0.167 calculated from inorganic salt analyses, then the activity coefficient of a univalent ion such as HCO_3^- would be 0.57 instead of 0.63, the value actually found and which is the value found for a salt solution of ionic strength $\mu = 0.167$. That it may play some rôle is not denied, but, in the absence of more exact knowledge of the effective valence type of the serum proteins, it does not seem profitable to attempt to evaluate the portion of calcium which is bound to the proteins in the non-ionized form, and the portion which represents the reduction in activity due to the ionic strength of the added protein.

XUM

Solubility of $CaCO_3$ in Transudates.

In a few instances analyses were made of edema fluid as drawn from the body. These results are given in Table XXII. They show that when allowance is made for the rôle played by the small amount of protein present, there is still a large discrepancy between the amount of calcium which is found and the value calculated for a salt solution which is in equilibrium with solid $CaCO_3$. These figures are given in the column headed [CaX] − [CaP].

TABLE XXII.

Calculation of $p([Ca] \times [CO_3^=])$ and [CaX] in Transudates, as Drawn.

Date.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Calculated $[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	[CaX] $\frac{M \times 10^3}{kg.\ H_2O}$	Protein $\frac{gm.}{kg.\ H_2O}$	Calculated* Ca proteinate $\frac{M \times 10^3}{kg.\ H_2O}$	[CaX] − [CaP]	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$
Mar. 29, 1923	7.46e	31.8	0.141	1.6	0.28	1.32	2.97	0.04	1.28	1.2	6.65
" 29, 1923	7.41e	31.8	0.125	2.0	0.32	1.68	26.6	0.38	1.30	1.2	6.60
May 8, 1923	7.45e	25.3	0.110	1.5	0.36	1.14	2.4	0.03	1.11		6.78
Sept. 21, 1923	7.47e	38.20	0.174	1.41	0.23	1.18	3.0	0.04	1.14	1.0	6.61
Dec. 1, 1925	7.46c	32.26	0.143	2.30	0.28	2.02	33.0	0.48	1.54	1.3	6.49
Sept. 29, 1924 After saturation with $CaCO_3$ at 38°.	7.12c	31.03	0.0617	1.98	0.65	1.33	15.5	0.17	1.16		

*Ca proteinate calculated from the equation Ca proteinate = 0.01 protein $(p\alpha_{H^+} - 6.0)$.

In order to determine whether edema fluid is in equilibrium with solid $CaCO_3$ it was equilibrated with solid $CaCO_3$. The calculated amount of [CaX] − [CaP] was 1.16 mM, showing that there had been no measurable loss in this moiety of the calcium due to the saturation. This fraction of the calcium, as we have stated before, is considered to be calcium in the non-ionic form, but whether it is unprecipitated $Ca_3(PO_4)_2$ or $CaHPO_4$, or is an un-

dissociated calcium organic compound, it would be impossible to decide from these experiments. It should be pointed out in connection with the above experiments, that the values of [CaX] − [CaP] are approximately equimolar with the concentration of phosphate.

Effect of Parathormone on $CaCO_3$ Solubility.

Since extirpation of the parathyroid glands results in such marked reduction in the calcium concentration of serum, it was obvious that one should attempt to demonstrate an influence of the parathyroid hormone prepared by Collip upon the solubility of $CaCO_3$ in salt solutions and in serum.

TABLE XXIII.

Effect of Parathormone upon Solubility of $CaCO_3$ in Salt Solutions, $\mu = 0.175$.

Solutions saturated 20 hours with solid $CaCO_3$ at 38°.

Experiment No.	Concentration of parathormone cc. per 100.	Initial calcium concentration $\frac{M \times 10^3}{kg.\ H_2O}$	$p_{H}H^+$	$[CO_3] \frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=] \frac{M \times 10^3}{kg.\ H_2O}$	$[Ca] \frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$
1	0.0	3.25	7.33c	19.77	0.0642	0.93	7.22
2	2.0	3.25	7.47c	29.80	0.1356	2.18	6.53
3	0.0	0.0	7.53c	31.84	0.1672	0.56	7.03
4	2.0	0.0	7.36c	22.09	0.0771	0.62	7.33

This has been attempted, but, although an effect of the parathyroid hormone upon the solvent power of serum for $CaCO_3$, has apparently been demonstrated, it is appreciated that the concentration of extract in our solutions was many times the concentration which is effective in raising the calcium of the blood *in vivo*. Therefore, in spite of the fact that the following experiments seem to indicate that this substance aids in the retention of calcium in solution, it is not felt that it, and it alone, constitutes the unknown organic substance which combines with calcium in the blood.

In the first experiments, salt solutions of serum ionic strength containing no calcium initially, but containing some of Collip's

preparation, were equilibrated with solid $CaCO_3$. No more $CaCO_3$ was dissolved than would have been dissolved by the salt solutions alone, as Table XXIII shows. When the same salt-hormone solutions contained calcium in solution initially, saturation with solid $CaCO_3$ did not result in precipitation of $CaCO_3$ as it did in the case of salt solutions containing no hormone. Whether this effect can be attributed entirely to the hormone or to the presence of some impurity, it is impossible to say. Nor do we know what is the limiting amount of calcium which it would be possible for the extract to hold in solution. These are questions upon which further work must be done.

An experiment similar to the above, except in two respects, namely that a small amount of phosphate as well as calcium was initially in solution, and the saturating phase consisted of $Ca_3(PO_4)_2$ as well as $CaCO_3$, was also performed. In this case the calcium was precipitated to the same level in the presence as in the absence of the extract.

Although these experiments do not prove that the parathyroid hormone plays a significant part in maintaining the non-ionized moiety of calcium in the body normally, they do constitute evidence that a substance of biological origin may act like citric acid in leading to what appears to be supersaturation with respect to calcium carbonate under conditions in which supersaturation is apparently precluded as a possibility.

Solubility of $CaCO_3$ in Serum of Parathyroidectomized Dogs.

Another argument against the hypothesis that the internal secretion of the parathyroid glands is the X factor in the blood which is combined with calcium in the unionized form, is found in the following series of experiments.

After removing a sample of blood for control analyses, thyreoparathyroidectomy was performed on a dog under ether anesthesia and with aseptic technique. The operation was performed by Dr. Douglas Boyd to whom we are greatly indebted. Samples of blood were withdrawn for analysis of the CO_2, $p\alpha_{H^+}$, and Ca at various times subsequent to the operation. Well marked tetany developed on the 2nd day following the operation, which was relieved by the intravenous injection of a 20 per cent solution

of $CaCl_2$. An attack of tetany recurred on the following day and was again relieved by the intravenous injection of $CaCl_2$. This was repeated several times during the course of 2 weeks.

The point of particular interest to us was to determine whether $CaCO_3$ could be held in solution by the blood serum when the dog was in tetany and its calcium, as is well known, was markedly below normal. Three experiments upon this point, all of which showed the same result, were performed. The protocol of one

TABLE XXIV.

Solubility of $CaCO_3$ in Serum of a Dog Following Parathyroidectomy.

Serum No.	$p\alpha_{H^+}$	$[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$p([Ca] \times [CO_3^=])$	Remarks.
1	7.43c	29.30	0.1213	2.73	6.48	Anteoperative. Serum analyzed as drawn.
2	7.42c	27.76	0.115	1.28	6.83	5 days postoperative. Marked tetany. Serum analyzed as drawn.
Serum 2 saturated with $CaCO_3$ at different $p\alpha_{H^+}$ values after augmenting calcium concentration to 3.2 mM with $CaCl_2$.						
2a	7.26	28.67	0.0782	3.14	6.61	
2b	7.52	26.00	0.1333	2.96	6.41	
2c	7.67	24.17	0.1765	2.87	6.29	

such experiment is given in Table XXIV. It illustrates that although the serum calcium had fallen to 1.28 mM of calcium per kilo of water, nevertheless, even after saturation with $CaCO_3$, the serum was fully capable of holding calcium in solution if it was added as $CaCl_2$.

DISCUSSION.

Our experiments upon $CaCO_3$ solubility in sodium chloride solutions provided us with the data necessary for the calculation

of the activity coefficient of calcium in salt solutions of varying ionic strength. In the case of citrate solutions it was found that either the presence of the citrate ion markedly affected the activity coefficient of the calcium ion or a slightly ionized calcium citrate compound was formed. The latter hypothesis seemed better substantiated by our data than the former.

In the above experiments, the same final state was reached whether one started with a higher or lower initial concentration of calcium than was required for equilibrium conditions. In experiments with serum these criteria of equilibria could not be successfully applied. Serum rotated with solid $CaCO_3$ showed no change in calcium concentration up to 24 hours[5] whether one started with a normal, high, or low initial calcium concentration. The amount of CaX[6] in serum, *i.e.* the amount present in excess

[5] The calcium of serum drops after 48 hours but concomitantly the inorganic phosphate rises indicating that certain splitting of organic phosphate compounds is occurring. This may account for the drop in serum calcium found by Irving (1926) after shaking serum with $CaCO_3$.

[6] Although the behavior of strong electrolytes has been satisfactorily accounted for by the theory of complete dissociation and the mathematical treatment accorded this theory by Debye and Hückel, the conformity of weak electrolytes to this theory has not yet been as thoroughly demonstrated. If one is dealing with a substance in solution which is completely dissociated and if one can measure the thermodynamic activity of its ions and their stoichiometric concentration, then the activity divided by the concentration equals the activity coefficient; *e.g.*,

$$Ag^+ + Cl^- \rightleftharpoons AgCl$$

$$\frac{\alpha_{Ag^+}}{[Ag^+]} = \gamma_{Ag^+} \text{ and } \frac{\alpha_{Cl^-}}{[Cl^-]} = \gamma_{Cl^-}$$

In this case the silver ion concentration and total silver concentration are identical.

If, on the other hand, one has a solution of the complex potassium silver cyanide, $KAg(CN)_2$, which dissociates but slightly into Ag^+ ions how should one define the activity coefficient of the silver ion?

$$KAg(CN)_2 \rightleftharpoons K^+ + Ag(CN)_2^-$$
$$\updownarrow$$
$$Ag^+ + 2(CN)^-$$

of that accounted for by the salts of serum, is only partially the result of the serum proteins. At $p\alpha_{H^+}$ 7.40 approximately 0.5 mM is due to the salts and 1.0 mM due to the proteins, leaving a residuum of 1.0 mM unaccounted for. These facts might be interpreted either on the hypothesis (*a*) that a portion of the calcium exists in serum in non-ionic form, or (*b*) that there is a delayed equilibrium between the calcium and carbonate in the serum.

According to the first hypothesis the calcium could conceivably exist as unprecipitated $CaCO_3$, $CaHPO_4$, or $Ca_3(PO_4)_2$, or as a non-ionized calcium compound. The possibility that it is unprecipitated $CaCO_3$ seems precluded by the fact that the same values of pK_1' of H_2CO_3 are obtained in normal serum and serum from which the calcium has been removed. This would not be the case if unprecipitated $CaCO_3$ were present. Nor does the

---

Will it be

$$(a)\ \frac{\alpha_{Ag^+}}{[Ag^+]} = \gamma_{Ag^+} \text{ or } (b)\ \frac{\alpha_{Ag^+}}{[Ag]} = \gamma_{Ag^+}$$

where $[Ag^+]$ represents the molal concentration of the silver ions and $[Ag]$ equals the molal concentration of total silver in the solution? In this example the silver ion concentration is only a fraction of the total silver concentration. In the treatment of the calcium systems with which we are dealing we have used the activity coefficient as defined by expression (*a*); namely,

$$\frac{\alpha_{Ca^{++}}}{[Ca^{++}]} = \gamma_{Ca^{++}}$$

For the numerical value of $\gamma_{Ca^{++}}$ in serum we have used that value found in a salt solution of the same ionic strength (the proteins being regarded as monovalent). From $\gamma_{Ca^{++}}$ and the value calculated for $\alpha_{Ca^{++}}$, the value of $[Ca^{++}]$ has been calculated. This, when subtracted from the total Ca gives what we have termed the unionized calcium, [CaX]. It should be noted that CaX does not represent a single chemical individual however. If it should be subsequently proven that the calcium compounds in serum are completely ionized, then this treatment would be incorrect and the activity coefficient of calcium in serum should be defined as $\alpha_{Ca^{++}}$ divided by [Ca], the total molal concentration of calcium in serum.

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existence of unprecipitated $CaHPO_4$ or $Ca_3(PO_4)_2$ seem to explain the facts because of the observation that serum shaken with $Ca_3(PO_4)_2$ results, not in the precipitation of calcium phosphate, but of calcium carbonate. The calculated amount of non-ionized calcium compound varies with the $p\alpha_{H^+}$ and the concentration of serum, but is independent of the length of time that serum is equilibrated with $CaCO_3$, providing no other change occurs in the serum.

The amount of residual calcium is as high in protein-free transudates as it is in serum. This suggests that the residual calcium is diffusible.

Against the first hypothesis is the fact that serum does not change its concentration of calcium when equilibrated with $CaCO_3$ after depletion or augmentation of its calcium concentration. It is realized that this is a real difficulty and at present is not understood.

According to the second hypothesis, a condition of delayed equilibrium exists in serum normally, and some factors are present which prevent the precipitation of $CaCO_3$. The fact that serum from which most of the inorganic phosphate has been removed becomes reversible with respect to $CaCO_3$ suggests that the phosphate of serum might be such an inhibiting factor. This experiment shows furthermore that there is no physical reason, such as coating of the particles, why $CaCO_3$ should not precipitate from serum. If, however, phosphate brings about a condition of apparent supersaturation of $CaCO_3$ in serum, it is difficult to understand why serum from which the calcium has been removed does not return to the initial condition of supersaturation, as it does in salt solutions containing phosphate. Additional evidence that there is no physical reason why $CaCO_3$ does not precipitate from serum will be given in the succeeding paper where it will be shown that shaking serum with solid $Ca_3(PO_4)_2$ causes the rapid precipitation of $CaCO_3$.

Further discussion will be postponed until the data of the succeeding papers have been given. It may be anticipated, however, that our work tends to illustrate the complexity of the system serum plus calcium salts and to throw doubt upon the possibility of describing the system in terms of any one factor.

SUMMARY.

1. A method has been described whereby the solubility product constant of calcium carbonate may be determined.

2. The method of calculating $CO_3^{=}$ ion concentration from analyses of $p\alpha_{H^+}$ and total $[CO_2]$ and of calculating $p\alpha_{H^+}$ from analyses of total $[CO_2]$ and CO_2 tension have been presented.

3. The stoichiometric solubility product of calcium carbonate has been found to vary with the ionic strength, according to the equation $pK'_{s.p.}\ CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.61\sqrt{\mu}}$.

4. The activity coefficient of Ca^{++} ion has been calculated from the above data, and found to vary with the ionic strength according to the equation $p\gamma_{Ca^{++}} = \frac{4.94\sqrt{\mu}}{1 + 1.61\sqrt{\mu}} - 1.6\sqrt{\mu}$.

5. The solubility of $CaCO_3$ in balanced salt solutions under various conditions has been studied, and the conditions for equilibrium determined.

6. Such solutions, when containing phosphate in solution, even in the presence of solid $CaCO_3$, indicated an apparent state of supersaturation with regard to that salt.

7. The solubility of $CaCO_3$ in solutions of sodium citrate was studied and was found to vary with the $p\alpha_{H^+}$ and the concentration of citrate. The formation of a slightly ionized calcium citrate compound seems indicated. The precipitation of calcium from a citrate solution saturated with $CaCO_3$, by equilibration with $Ca_3(PO_4)_2$ was demonstrated.

8. Analyses of serum as drawn indicate a stoichiometric solubility product for $CaCO_3$ of $10^{-6.40}$ compared with $10^{-7.40}$ in salt solutions of comparable ionic strength.

9. This value is unchanged by saturation of serum with solid $CaCO_3$ up to 22 hours.

10. Calcium carbonate did not dissolve in serum from which much of the calcium had been removed by oxalate.

11. Calcium carbonate did not precipitate from serum whose calcium concentration had been augmented by the addition of calcium chloride, even in the presence of solid $CaCO_3$.

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12. Serum appears to be reversible with respect to calcium salts following removal of the inorganic phosphates.

13. The effect of varying $p\alpha_{H^+}$ on the stoichiometric product, $p([Ca] \times [CO_3^{=}])$, has been studied.

14. The influence of varying the serum protein concentration on the product $p([Ca] \times [CO_3^{=}])$ was determined.

15. The solubility of $CaCO_3$ is transudates has been studied.

16. Parathormone was found to influence the solubility of $CaCO_3$ in salt solutions.

17. No evidence was found that the serum of parathyroidectomized dogs was unable to hold as much calcium in solution as normal serum.

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STUDIES OF THE SOLUBILITY OF CALCIUM SALTS.

II. THE SOLUBILITY OF TERTIARY CALCIUM PHOSPHATE IN SALT SOLUTIONS AND BIOLOGICAL FLUIDS.

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Continuing the study of the solubility of the bone salts under various conditions, this paper has for its purpose the presentation of such studies in systems where solid $Ca_3(PO_4)_2$ was the sole saturating body. The general experimental analytical procedure outlined in Paper I was followed throughout. The presence of another important anion, namely $PO_4^{\equiv}$ ion, necessitated the use of another set of ionization constants and new tables of calculations. The necessary exposition of the material used in calculating these experiments is given in the following section. The calculations based upon CO_2 analyses have been given in Paper I.

Calculations.

Calculation of $PO_4^{\equiv}$ Ion Concentration from Analyses of Total $[PO_4]$ and the $p\alpha_{H^+}$.

In calculating their results, Holt, La Mer, and Chown (1925), did not have data available that permitted them to distinguish between the stoichiometric or apparent dissociation constants of phosphoric acid, K_1', K_2', and K_3' and the constants at zero ionic strength, or the activity dissociation constants, K_1, K_2, and K_3. In order to avoid this difficulty, and to obtain the proper values to use at different ionic strengths, an attempt was made to obtain, either from the literature or from experiments, some idea as to how these constants varied with ionic strength. The present section has for its purpose an explanation of how such constants were obtained

* This paper is part of a thesis submitted by Julius Sendroy, Jr., in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

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and how they were used to calculate the concentrations of the three forms of phosphate in salt and serum calcium solubility product experiments.

Developing our equation in a manner similar to that adopted for calculating carbonate concentration, we have, from the mass law,

$$(1) \qquad \frac{\alpha_{H^+} \times \alpha_{H_2PO_4^-}}{\alpha_{H_3PO_4}} = K_1$$

$$(2) \qquad \frac{\alpha_{H^+} \times \gamma_{H_2PO_4^-} \times [H_2PO_4^-]}{\gamma_{H_3PO_4} \times [H_3PO_4]} = K_1$$

$$(3) \qquad \frac{\alpha_{H^+} \times [H_2PO_4^-]}{[H_3PO_4]} = \frac{K_1 \gamma_{H_3PO_4}}{\gamma_{H_2PO_4^-}} = K_1'$$

Similarly,

$$(4) \qquad \frac{\alpha_{H^+} \times [HPO_4^{=}]}{[H_2PO_4^-]} = \frac{K_2 \gamma_{H_2PO_4^-}}{\gamma_{HPO_4^{=}}} = K_2'$$

and

$$(5) \qquad \frac{\alpha_{H^+} \times [PO_4^{\equiv}]}{[HPO_4^{=}]} = \frac{K_3 \gamma_{HPO_4^{=}}}{\gamma_{PO_4^{\equiv}}} = K_3'$$

From equations (3), (4), and (5)

$$(6) \qquad [H_3PO_4] = \frac{(\alpha_{H^+})^3 \times [PO_4^{\equiv}]}{K_1' K_2' K_3'}$$

$$(7) \qquad [H_2PO_4^-] = \frac{(\alpha_{H^+})^2 \times [PO_4^{\equiv}]}{K_2' K_3'}$$

$$(8) \qquad [HPO_4^{=}] = \frac{\alpha_{H^+} \times [PO_4^{\equiv}]}{K_3'}$$

Hence, since

$$(9) \qquad \text{Total } [PO_4] = [H_3PO_4] + [H_2PO_4^-] + [HPO_4^{=}] + [PO_4^{\equiv}]$$

$$(10) \qquad \text{Total } [PO_4] = \frac{(\alpha_{H^+})^3 \times [PO_4^{\equiv}]}{K_1' K_2' K_3'} + \frac{(\alpha_{H^+})^2 \times [PO_4^{\equiv}]}{K_2' K_3'} + \frac{\alpha_{H^+} \times [PO_4^{\equiv}]}{K_3'} + [PO_4^{\equiv}]$$

Obtaining the concentrations of the mono-, di-, and trivalent phosphate ions in terms of the total phosphate, we have

$$(11)\quad [H_2PO_4^-] = \frac{\text{Total } [PO_4]\ (\alpha_{H^+})^2\ K_1'}{(\alpha_{H^+})^3 + (\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2' + K_1'\ K_2'\ K_3'}$$

$$(12)\quad [HPO_4^=] = \frac{\text{Total } [PO_4]\ \alpha_{H^+}\ K_1'\ K_2'}{(\alpha_{H^+})^3 + (\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2' + K_1'\ K_2'\ K_3'}$$

$$(13)\quad [PO_4^{\equiv}] = \frac{\text{Total } [PO_4]\ K_1'\ K_2'\ K_3'}{(\alpha_{H^+})^3 + (\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2' + K_1'\ K_2'\ K_3'}$$

For the calculations in this work, the magnitudes of the first and fourth terms of the denominator, $(\alpha_{H^+})^3$ and $K_1'\ K_2'\ K_3'$, are such that they may be neglected.

Thus equations (11), (12), and (13) may be simplified:

$$(14)\quad [H_2PO_4^-] = \frac{\text{Total } [PO_4]\ (\alpha_{H^+})^2\ K_1'}{(\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2'}$$

$$(15)\quad [HPO_4^=] = \frac{\text{Total } [PO_4]\ \alpha_{H^+}\ K_1'\ K_2'}{(\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2'}$$

$$(16)\quad [PO_4^{\equiv}] = \frac{\text{Total } [PO_4]\ K_1'\ K_2'\ K_3'}{(\alpha_{H^+})^2\ K_1' + \alpha_{H^+}\ K_1'\ K_2'}$$

When $[PO_4^{\equiv}]$ and $[HPO_4^=]$ are known, of course, $[H_2PO_4^-]$ is found from total $[PO_4]$ by difference, instead of by the laborious use of equation (14).

First Dissociation Constant of Phosphoric Acid.

From equation (3) we obtain, on taking the negative logarithm of all terms,

$$(17)\quad pK_1' = pK_1 + p\gamma_{H_2PO_4} - p\gamma_{H_2PO_4^-}$$

Since we have no data on the activity coefficient of H_3PO_4, it is assumed to be unity. Therefore, $p\gamma_{H_3PO_4}$ disappears, and we have

$$(18)\quad pK_1' = pK_1 - p\gamma_{H_2PO_4^-}$$

For the activity coefficient of an ion, in dilute solutions, Brönsted and La Mer (1924) have given the limiting equation developed as a consequence of the Debye-Hückel theory,

$$(19) \qquad -\log \gamma_{ion} = 0.5\, z_i^2 \sqrt{\mu}$$

where z_i is the valence of the ion. Hence, theoretically, in dilute solutions, we should have $p\gamma_{H_2PO_4^-} = 0.5\sqrt{\mu}$, and $pK_1' = pK_1 - 0.5\sqrt{\mu}$.

Examination of the literature revealed that very little work had been done on this constant. Noyes and Eastman (1907), by conductivity measurements, determined the effect of varying concentrations of phosphoric acid on the first dissociation constant

TABLE I.

First Dissociation Constant of Phosphoric Acid at 18°.

Recalculated from Noyes and Eastman's data given by Abbott and Bray (p. 749).

Formula weights per liter.	$K_1' \times 10^3$.	Corrected $K_1' \times 10^3$.	pK_1'	Approximate $\sqrt{\mu}$.
0.1	1.12	1.12	1.95	0.316
0.08	1.07	1.07	1.97	0.283
0.05	1.01	1.01	1.995	0.224
0.0125	0.90	0.90	2.045	0.112
0.01	0.88	0.88	2.055	0.100
0.002	0.71	0.81	2.09	0.045
0.0002	0.63	0.65	(2.185)*	0.014

* Not used in plotting the data.

at 18°, and their results were found recalculated in the work of Abbott and Bray (1909) (p. 749, Table X). Table I contains these values corrected for one or two errors, apparently of calculation. The ionic strength has not been calculated on the molality basis, but this is unnecessary here, inasmuch as the calculations are subject to much greater errors. The temperature is 20° lower than that for which we have adopted these figures, but the temperature coefficient of such weak acids in this range is probably not very great. Furthermore, the concentration was varied by increasing concentrations of H_3PO_4. There can be no doubt that the specific ion effect in such a solution may be quite different from that in one of NaCl of the same concentration.

However, although the values calculated by it may be greatly in error, we have adopted for our calculation the equation which seems best fitted to suit the data, as affording some indication of the variation in magnitude of this constant between infinite dilution and the salt concentrations used in these experiments.

The data have been plotted in Fig. 1 with pK_1' as ordinates and $\sqrt{\mu}$ as abscissæ. Extrapolation to $\sqrt{\mu} = 0$ gives the empirical equation which fits the data, as

$$pK_1' = 2.11 - 0.5\sqrt{\mu} \tag{20}$$

$$\text{Hence, } p\gamma_{H_2PO_4^-} = 0.5\sqrt{\mu} \tag{21}$$

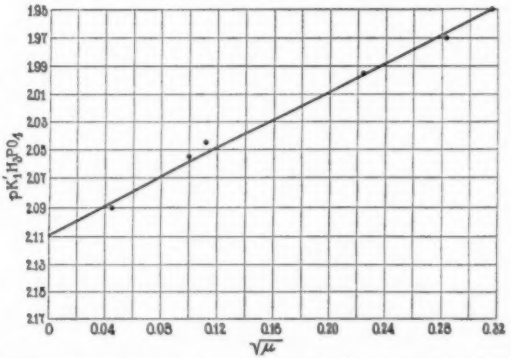

FIG. 1. The variation in the first dissociation constant of H_3PO_4 with ionic strength. Values of pK_1' are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The equation of the solid line is $pK_1' = 2.11 - 0.5\sqrt{\mu}$.

Abbott and Bray give $K_1' = 1.1 \times 10^{-2}$ at a normality of about 0.09, or $\sqrt{\mu} = 0.3$. Accordingly, using $p\gamma_{H_2PO_4^-} = 0.5\sqrt{\mu}$, pK_1 would be 2.12 which is in good agreement with Noyes and Eastman's data. For salt solutions at $\sqrt{\mu} = 0.390$ and for serum, we have used the value $K_1' = 1.22 \times 10^{-2}$ or $pK_1' = 1.915$.

Second Dissociation Constant of Phosphoric Acid.

From equation (4) we obtain

$$pK_2' = pK_2 + p\gamma_{H_2PO_4^-} - p\gamma_{HPO_4^=} \tag{22}$$

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The Brönsted-La Mer equation given above would make $p\gamma_{HPO_4^=} = 2.0\sqrt{\mu}$. Hence in dilute solutions, pK_2' should = $pK_2 - 1.5\sqrt{\mu}$.

For this constant various authors have given values in the literature. Apparently the stoichiometric dissociation constant pK_2' is greatly affected by the medium in which it is determined. Unpublished experiments done in this laboratory give as a first approximation, pK_2' at 20° = $7.18 - 1.25\sqrt{\mu}$ where the ionic strength was varied by increasing concentrations of NaCl. The pK_2' was determined by the equation

$$p\alpha_{H^+} = pK_2' + \log \frac{[HPO_4^=]}{[H_2PO_4^-]} \tag{23}$$

Varying proportions of the monobasic and dibasic phosphate were mixed and $p\alpha_{H^+}$ determined electrometrically. Assuming that the temperature coefficient of 0.03 obtained by Hastings and Sendroy (1924) holds for all dilutions,

$$pK_2' \text{ at } 38° = 7.15 - 1.25\sqrt{\mu} \tag{24}$$

and

$$p\gamma_{HPO_4^=} = 1.75\sqrt{\mu} \tag{25}$$

We have used values for pK_2' based on these formulas in all of our phosphate calculations.

Michaelis and Kruger (1921) and Michaelis and Garmendia (1914) have also published results with which our data agree very well. Their values for pK_2' in solutions of varying sodium chloride, phosphate, and sulfate content, indicate a difference in activity coefficient of the dibasic phosphate ion due to specific ion effects. However, inasmuch as our experimental salt solutions and serum contain a predominance of NaCl over the concentration of the other salts present, we have taken our value for pK_2' at different ionic strengths as determined by the results with solutions of NaCl, and have used the constants so obtained in calculating all phosphate determinations.

Abbott and Bray (p. 755, Table XVII) give a value, when recalculated, of $pK_2' = 6.73$ at 38° for $\sqrt{\mu} = 0.55$ in a solution of sodium ammonium phosphate salts. This value fits in with the data cited before and also with our own.

For salt solutions at $\sqrt{\mu} = 0.390$ and for serum, we have used the value $K_2' = 2.19 \times 10^{-7}$ or $pK_2' = 6.66$.

Third Dissociation Constant of Phosphoric Acid.

From equation (5) we obtain

$$pK_3' = pK_3 + p\gamma_{HPO_4^=} - p\gamma_{PO_4^{\equiv}} \tag{26}$$

The Brönsted-La Mer equation would make $p\gamma_{PO_4^{\equiv}} = 4.5\sqrt{\mu}$, and $pK_3' = pK_3 - 2.5\sqrt{\mu}$. For this constant, Abbott and Bray, Blanc (1920), Brydges (1911), and Prideaux and Ward (1924) have given different values ranging from 3.6×10^{-13} to 1×10^{-12}. Most of these results were obtained at room temperature and under various experimental conditions. However, no information could be found upon the variation of the third stoichiometrical dissociation constant with varying ionic strength. Accordingly, in order to obtain some idea of the effect of varying concentration on the activity of $PO_4^{\equiv}$ ion, an attempt was made to obtain the necessary values at 38° experimentally. This was done by determining the $p\alpha_{H^+}$ and the concentration of tribasic and dibasic phosphate ions in solutions whose ionic strength was varied by NaCl. Since it was impossible to work in CO_2-free solutions, the CO_2 content was also determined and correction made for the amount of Na_2CO_3 and $NaHCO_3$ present; correction was also made for the concentration of NaOH. These results have been plotted in Fig. 2 with pK_3' as ordinates and $\sqrt{\mu}$ as abscissæ. The empirical equation approximately fitting these data is

$$pK_3' = 12.66 - 2.25\sqrt{\mu} \tag{27}$$

from which

$$p\gamma_{PO_4^{\equiv}} = 4.0\sqrt{\mu} \tag{28}$$

Although the points determining the line which fits the above equation lie between concentrations $\sqrt{\mu} = 0.25$ and 0.50, and it is realized that the extrapolation to zero ionic strength involves an error of considerable magnitude, in the absence of better data we have been forced to use the above value for all phosphate calculations. Most of the experiments on salt solutions, and certainly those on serum, have concentrations between the above

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limits. For salt solutions at $\sqrt{\mu} = 0.390$ and for serum we have used the value $K_3' = 1.66 \times 10^{-12}$ or $pK_3' = 11.78$.

It is believed, that while the three constants for phosphoric acid derived in this section may be quite in error, particularly K_1 and K_3, nevertheless, they seem the best available at the present time.

EXPERIMENTAL RESULTS.

Solubility of $Ca_3(PO_4)_2$ in Salt Solutions.—In view of the thorough study made by Holt, La Mer, and Chown, we have made no attempt to study aqueous systems in equilibrium with $Ca_3(PO_4)_2$, as completely as those in equilibrium with $CaCO_3$ and with both

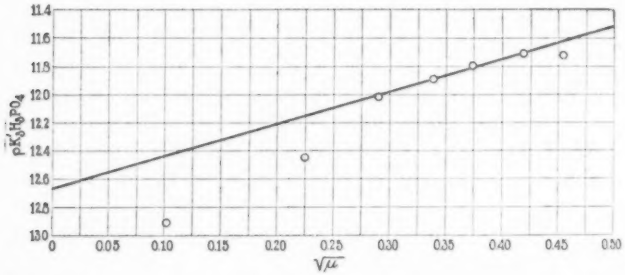

FIG. 2. The variation in the third dissociation constant of H_3PO_4 with ionic strength. Values of pK_3' are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The equation of the solid line is $pK_3' = 12.66 - 2.25\sqrt{\mu}$.

salts. In order that our picture of the various systems might be complete, however, and the results comparable with each other, a few experiments in which only solid $Ca_3(PO_4)_2$ was the solid phase have been performed.

Holt, La Mer, and Chown studied the solubility of $Ca_3(PO_4)_2$ in salt solutions of varying ionic strength. Using the constants for phosphoric acid which we have in part determined and in part obtained from the literature, we have recalculated their data in order that our experimental results may be compared with theirs. These experiments are discussed in Paper III in connection with the variation in solubility product of $Ca_3(PO_4)_2$ with ionic strength.

The aqueous systems in which $pK'_{s.p.}$ $Ca_3(PO_4)_2$ was determined all contained salts in amount and kind comparable to those found

in serum and transudates. The solutions were equilibrated at 38° from 20 hours to 8 days with solid $Ca_3(PO_4)_2$ at definite CO_2 tensions. At the end of the saturation period, the liquid phase was analyzed for CO_2, total phosphate, calcium, and $p\alpha_{H^+}$. From these results were calculated $[CO_3^=]$ and $[PO_4^{\equiv}]$ concentrations. Each of these, when multiplied by the calcium ion concentration, is the apparent solubility product for the salt studied. The results are given in Table II. In all of these experiments the

TABLE II.

Solubility of $Ca_3(PO_4)_2$ in Salt Solutions Saturated at 38° with Solid $Ca_3(PO_4)_2$.

Experiment No.	Saturation time.	Initial concentrations.			Final concentrations.		
		$[HCO_3^-]$ M × 10^3 / kg. H_2O	Total $[PO_4]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	Total $[CO_2]$ M × 10^3 / kg. H_2O	$[HCO_3^-]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O
7	20 hrs.	30.27	0.0	0.0	27.14	25.58	0.097
10	8 days.	30.27	0.0	0.0	28.53	27.17	0.130
6	20 hrs.	30.27	0.0	7.76	18.17	16.64	0.041
4	20 "	30.27	1.21	0.0	25.66	24.19	0.094
3	20 "	35.32	1.21	4.65	25.05	23.48	0.079

	Final concentrations.				$pK'_{s.p.}$ $CaCO_3$	$pK'_{s.p.}$ $Ca_3(PO_4)_2$	$\sqrt{\mu}$
	Total $[PO_4]$ M × 10^3 / kg. H_2O	$[PO_4^{\equiv}]$ M × 10^3 / kg. H_2O	$[Ca^{++}]$ M × 10^3 / kg. H_2O	$p\alpha_{H^+}$			
7	1.97	6.42	0.05	7.37	8.31	27.28	0.381
10	0.96	4.07	0.03	7.47	8.41	28.34	0.385
6	0.126	0.242	0.59	7.18	7.62	26.93	0.376
4	3.69	12.36	0.04	7.38	8.43	27.00	0.379
3	2.03	5.79	0.04	7.32	8.50	27.68	0.384

final concentration of calcium was so low that the results are not as reliable as those of our other experiments. However, it is quite apparent that in Experiments 4, 7, and 10, in which no calcium was originally present, the amount of calcium in solution at equilibrium was not sufficient to exceed the solubility product of $CaCO_3$. The values of $pK'_{s.p.}$ $Ca_3(PO_4)_2$, however, are more nearly of the order of magnitude to be expected in solutions of this ionic strength.

Experiment 4 is of particular interest because although the initial phosphate concentration was 1.21 mM, the final concentration was 3.69, indicating that 1.24 mM $Ca_3(PO_4)_2$ had gone into solution. Had none of the calcium precipitated, there would have been 3.72 mM calcium present at the end of the experiment, whereas analysis showed only 0.04 mM. If this calcium had precipitated as $CaCO_3$, there would have been a fall of 7.36 mM in $[HCO_3^-]$. The fall was actually 6.11 mM. This suggests that possibly some of the $Ca_3(PO_4)_2$ reacted with NaH_2PO_4 in the solution with the resulting precipitation of $CaHPO_4$.

In Experiments 6 and 3, calcium was initially present in solution; phosphate was initially present in solution in Experiment 3, but not in No. 6. After 20 hours saturation, most of the calcium had been precipitated and 0.126 mM of phosphate had been dissolved. The product $pK'_{s.p.}$ $Ca_3(PO_4)_2$ was nearly normal but $pK'_{s.p.}$ $CaCO_3$ was high, indicating unsaturation.

All of the values for $pK'_{s.p.}$ $Ca_3(PO_4)_2$ were higher than the corresponding values in salt solutions saturated with both solids. In the main, these experiments illustrate the greater difficulty which is encountered in bringing to equilibrium a system involving $Ca_3(PO_4)_2$, particularly if one starts from zero calcium concentration. Under these conditions, the solubility product of $CaCO_3$ was not equalled or exceeded.

These results are in marked contrast to similar experiments in which only $CaCO_3$ was the saturating body. Under such conditions, saturation of the solution with $CaCO_3$ resulted in apparent *supersaturation* in the case of *both* $CaCO_3$ and $Ca_3(PO_4)_2$ when phosphate was initially present in the solution, regardless of the initial calcium content. Here, saturation with solid $Ca_3(PO_4)_2$ for as long as 8 days did not result in the solubility product of $CaCO_3$ being equalled. Furthermore, even when an excess of calcium was initially present, the solution was still unsaturated with respect to $CaCO_3$. Inasmuch as the solutions of this section were not equilibrated in the presence of solid $CaCO_3$, results obtained for $pK'_{s.p.}$ were not contrary to anything that might have been expected for such systems.

Solubility of $Ca_3(PO_4)_2$ in Serum.—From analyses of the Ca, inorganic phosphate, and $p\alpha_{H^+}$ of serum as drawn, one may

calculate the product $[Ca]^3 \times [PO_4^{\equiv}]^2$.* In horse serum at $p\alpha_{H^+} = 7.40$ this is approximately 22.50 when expressed as $p([Ca]^3 \times [PO_4^{\equiv}]^2)$ and agrees well with values found by Holt, La Mer, and Chown when recalculated with the use of the constants given in the previous section (see Table III). This represents a high degree of apparent supersaturation over the value found at equilibrium in salt solutions of the same ionic strength.

TABLE III.

$p([Ca]^3 \times [PO_4^{\equiv}]^2)$ of Serum before and after Shaking with $Ca_3(PO_4)_2$ at 38°.

Recalculated data of Holt, La Mer, and Chown (p. 575).

$p\alpha_{H^+}$	[Ca] M × 10³ kg. H_2O	Total $[PO_4]$ M × 10³ kg. H_2O	$[PO_4^{\equiv}]$ M × 10⁶ kg. H_2O	$pPO_4^{\equiv}$	pCa	$pCa_3(PO_4)_2$
I. Human T. C. G. H_2O assumed, 930 gm. per liter.						
7.40	2.72	1.11	3.92	7.407	2.565	22.51
7.70	0.484	0.520	3.96	7.402	3.349	24.84
II. Human C. O'D. H_2O assumed, 930 gm. per liter.						
7.40	2.74	1.22	4.31	7.365	2.562	22.42
7.70	0.672	0.503	3.83	7.417	3.298	24.73
III. Horse 5515-H. H_2O assumed, 940 gm. per liter.						
7.35	2.93	1.17	3.61	7.443	2.533	22.49
7.40	0.718	0.720	2.54	7.595	3.144	24.62

Thus $Ca_3(PO_4)_2$ as in the case of $CaCO_3$, is present in serum as drawn in a condition of apparent supersaturation. Unlike the behavior of serum in the presence of $CaCO_3$, however, saturation with $Ca_3(PO_4)_2$ results in the precipitation of calcium. Holt, La Mer, and Chown showed this to be the case for human and horse serum by analyses of the serum before and after saturation

* Since $pK'_{s.p.}\ Ca_3(PO_4)_2 = 3\ pCa^{++} + 2\ pPO_4^{\equiv}$, it is erroneous to use $pK'_{s.p.}\ Ca_3(PO_4)_2$ for the term $3\ pCa + 2\ pPO_4^{\equiv}$. Here Ca denotes the *total* calcium, and until it can be definitely proved that this quantity is all ionized calcium, the use of $pK'_{s.p.}\ Ca_3(PO_4)_2$ should be carefully restricted. For convenience, we shall hereafter refer to $p([Ca]^3 \times [PO_4^{\equiv}]^2)$ as $p\ Ca_3(PO_4)_2$ and $p([Ca] \times [CO_3^{=}])$ as $pCaCO_3$.

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with $Ca_3(PO_4)_2$ for a period of 1 to 2 weeks. In their three experiments (Table III), the calcium decreased 2.24, 2.08, 2.21 mM per kilo of H_2O; correspondingly, the total inorganic phosphate decreased 0.59, 0.72, 0.45 mM. These data show that the calcium could not have been precipitated entirely as $CaHPO_4$ or even as $Ca_3(PO_4)_2$ which has the highest Ca:P ratio. Calculated with our constants, the value of $p([Ca]^3 \times [PO_4^{\equiv}]^2)$ or $pCa_3(PO_4)_2$ = 24.84, 24.73, and 24.62, respectively, after saturation as compared with the value 22.50 before saturation.

We have confirmed this result in the following manner. Horse serum was equilibrated with $Ca_3(PO_4)_2$ at a tension designed to keep the $p\alpha_{H^+}$ approximately 7.4. After varying intervals of time, samples were removed for analysis of the CO_2, inorganic

TABLE IV.

Effect of Saturating Horse Serum at 38° with Solid $Ca_3(PO_4)_2$ for Varying Lengths of Time.

Experiment No.	Saturation time.	$p\alpha_{H^+}$ (colorimetric).	Total $[CO_2]$ M × 10^3 / kg. H_2O	$[HCO_3^-]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	Total $[PO_4]$ M × 10^3 / kg. H_2O	$[PO_4^{\equiv}]$ M × 10^3 / kg. H_2O	[Ca] M × 10^3 / kg. H_2O	$pCaCO_3$	$pCa_3(PO_4)_2$
1	0	7.47	32.32	30.78	0.147	0.787	3.33	3.19	6.33	22.46
2	5 min.	7.28	28.91	26.96	0.083	0.904	2.31	0.74	7.21	24.66
3	1 hr.	7.37	27.13	25.57	0.097	0.815	2.66	0.62	7.22	24.78
4	3½ hrs.	7.37	27.39	25.80	0.098	0.860	2.80	0.62	7.23	24.77

phosphate, Ca, and $p\alpha_{H^+}$. The results of this experiment are given in Table IV. It is seen that the greatest precipitation of calcium took place within the first 5 minutes and was practically complete at the end of 1 hour. Longer saturation failed to bring about further precipitation of calcium.

The form in which it precipitated under these conditions is a matter of great interest. As there was little or no change in the inorganic phosphate during the experiment, the possibility of the calcium having been precipitated as a phosphate seems precluded. There was, however, a marked decrease in the bicarbonate concentration which quantitatively accounts for the precipitation of the calcium as $CaCO_3$.

In the first interval the decrease in calcium was 2.45 mM. If it precipitated as $CaCO_3$, the $[HCO_3^-]$ concentration would have

fallen 4.90. Allowing for the change in $p\alpha_{H^+}$, the decrease in $[HCO_3^-]$ was 4.66 mM. Similarly, in the next interval one may calculate from the decrease in calcium, an expected fall of 0.24 in $[HCO_3^-]$. By analysis the decrease was 0.35 mM. It would seem then, that one cannot be dealing here with a system supersaturated with respect to $Ca_3(PO_4)_2$ in the usual sense of the term.

This result is quite comparable to that obtained with the sodium citrate solutions mentioned in Paper I. There, the citrate solution was first brought into equilibrium with $CaCO_3$. Upon exchanging the $CaCO_3$ for $Ca_3(PO_4)_2$ as the saturating body, calcium carbonate was precipitated. This illustrates a behavior of citrate solutions toward calcium salts, analogous to that of serum. The significance of these results will be discussed in the following paper.

SUMMARY.

1. From data in the literature, the equation relating the first dissociation constant of phosphoric acid to the ionic strength of the solution has been found to be $pK_1' = 2.11 - 0.5\sqrt{\mu}$ at 18°. This value was applied to our work done at 38°.

2. From our own data, and from that in the literature, the equation relating the second dissociation constant of phosphoric acid to the ionic strength of the solution has been found to be $pK_2' = 7.15 - 1.25\sqrt{\mu}$ at 38°.

3. From our own data, the equation relating the third dissociation constant of phosphoric acid to the ionic strength has been found to be $pK_3' = 12.66 - 2.25\sqrt{\mu}$ at 38°.

4. The solubility of $Ca_3(PO_4)_2$ in salt solutions under various conditions has been studied.

5. The solubility of $Ca_3(PO_4)_2$ in serum has been studied. The results indicate a rapid precipitation of $CaCO_3$ when solid $Ca_3(PO_4)_2$ is added to serum.

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STUDIES OF THE SOLUBILITY OF CALCIUM SALTS.

III. THE SOLUBILITY OF CALCIUM CARBONATE AND TERTIARY CALCIUM PHOSPHATE UNDER VARIOUS CONDITIONS.

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It is well known that the two primary bone salts are $CaCO_3$ and $Ca_3(PO_4)_2$, with more or less $CaHPO_4$. Probably the best available figures for the amount of $CaCO_3$ in bone are those of Goto (1918) whose analyses for calcium and for carbonate by an improved method of Van Slyke (1918) give values in bone of normal rabbits of 14.3 per cent calcium and 5.0 per cent CO_2 (carbonate 6.8 per cent). The figures for phosphate cannot be used because they include the organic phosphate of the marrow lipoids. Calcium carbonate would evidently be 11.3 per cent, and tertiary calcium phosphate, if all of the remaining calcium be assumed in that form, would be 25.3 per cent, giving a $CaCO_3$: $Ca_3(PO_4)_2$ ratio of 31 per cent : 69 per cent. Although some carbonate may have been present as the sodium salt, this is nevertheless a decidedly higher figure than the 15 : 85 per cent ratio commonly quoted for the bone salts, and places somewhat more stress on the importance of calcium carbonate in calcium metabolism.

In the absence of evidence as to the presence of $CaHPO_4$ in bone, the study of the solubility of the calcium salts of bone was confined to $CaCO_3$ and $Ca_3(PO_4)_2$. Having obtained the results of the preceding paper for tricalcium phosphate under various conditions, and in view of the results of Holt, La Mer, and Chown (1925 *a*, *b*) which appeared while the study of calcium carbonate

* This paper is part of a thesis submitted by Julius Sendroy, Jr., in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

equilibria was in progress, it seemed desirable, since bone contains solid $CaCO_3$ and $Ca_3(PO_4)_2$, to ascertain the equilibrium conditions for salt solutions and serum in the presence of these two solids. *A priori*, one would expect that equilibrium for both, that is a condition of saturation for both salts, would only be obtained in the presence of the two salts as saturating bodies. The experiments performed clearly indicate, with a few exceptions that seem fairly well capable of explanation, that such is the case.

In accordance with the theoretical considerations given, for any salt solution in equilibrium with solid $CaCO_3$ and $Ca_3(PO_4)_2$, the following equations hold:

$$(1) \qquad \alpha_{Ca^{++}} \times \alpha_{CO_3^{=}} = K_{s.p.\ CaCO_3}$$

$$(2) \qquad (\alpha_{Ca^{++}})^3 \times (\alpha_{PO_4^{\equiv}})^2 = K_{s.p.\ Ca_3(PO_4)_2}$$

Eliminating $\alpha_{Ca^{++}}$, one obtains

$$(3) \qquad \frac{\alpha_{CO_3^{=}}}{(\alpha_{PO_4^{\equiv}})^{\frac{3}{2}}} = \frac{K_{s.p.\ CaCO_3}}{K_{s.p.\ (Ca_3(PO_4)_2)^{\frac{1}{2}}}}$$

For any solution of fixed ionic strength,

$$(4) \qquad \frac{[CO_3^{=}]}{[PO_4^{\equiv}]^{\frac{3}{2}}} = \frac{K'_{s.p.\ CaCO_3}}{K'_{s.p.\ (Ca_3(PO_4)_2)^{\frac{1}{2}}}}$$

or expressed in terms of negative logarithms

$$(5) \qquad pCO_3^{=} - \frac{2pPO_4^{\equiv}}{3} = pK'_{s.p.\ CaCO_3} - \frac{pK'_{s.p.\ Ca_3(PO_4)_2}}{3}$$

This equation shows that in any solution in equilibrium with both solid salts, $CaCO_3$ and $Ca_3(PO_4)_2$, there will be a definite ratio between the $CO_3^{=}$ ion concentration and the $PO_4^{\equiv}$ ion concentration; and one and only one ratio is compatible with equilibrium for both salts. Furthermore, fixing either the $[CO_3^{=}]$ or the $[PO_4^{\equiv}]$ fixes the $[Ca^{++}]$ which can be in equilibrium with the solid phase at that ionic strength.

One may fix the system in any one of a variety of ways. We have centered our attention on fixing the $p\alpha_{H^+}$ within physiological limits by adjusting the CO_2 tension. This has fixed the $[CO_3^{=}]$ which in turn fixed the $[Ca^{++}]$. Where phosphate was also

present, there must have been a further adjustment until the ratio of $\frac{[CO_3^{=}]}{[PO_4^{\equiv}]^{\frac{2}{3}}}$ satisfied the requirements of equilibrium.

EXPERIMENTAL RESULTS.

Effect of Varying Ionic Strength on Apparent or Stoichiometric Solubility Products of $CaCO_3$ and $Ca_3(PO_4)_2$.

Salt solutions were made up as outlined in the previous papers. Each solution contained calcium, phosphate, and bicarbonate with 1 per cent of volume added in weight as $CaCO_3$ and $Ca_3(PO_4)_2$. Eight different solutions were prepared, the ionic strength being increased in the second and third by $NaHCO_3$, after which the $NaHCO_3$ concentration was kept constant and the ionic strength increased with NaCl. The experiment was repeated (Series A) by preparing eight new solutions. Calcium was analyzed in 5 cc. samples and titrated with N/200 $KMnO_4$. The results given by electrometric $p\alpha_{H^+}$ determinations were all so unsatisfactory, apparently due to the electrode "poisoning" encountered by Holt, La Mer, and Chown, that the $p\alpha_{H^+}$ was calculated on the basis of total CO_2 and CO_2 tension analyses as outlined in the section on calculations.

The analytical determinations of calcium gave the value of $[Ca^{++}]$ immediately, since the difficultly soluble salts are assumed to be completely ionized. For each of the sixteen solutions, $p\alpha_{H^+}$ was calculated by equation (50), Paper I, by using the proper values for α_{CO_2} in accordance with equation (48). From the total $[CO_2]$, values for $[CO_3^{=}]$ and $[HCO_3^{-}]$ were obtained by equations (32) and (33), respectively. From the total $[PO_4]$ values for $[PO_4^{\equiv}]$ and $[HPO_4^{=}]$ were obtained by equations (16) and (15), Paper II, respectively.

Tables I and II contain the data obtained in these experiments. They have been plotted in Figs. 1 and 2 with $pK'_{s.p.}CaCO_3$ and $pK'_{s.p.}Ca_3(PO_4)_2$ as ordinates, and $\sqrt{\mu}$ as abscissæ. The equation of the line through these points was obtained in the following manner.

In order to calculate $pK'_{s.p.}$ of $CaCO_3$ and $Ca_3(PO_4)_2$ accurately, it was necessary to employ the stoichiometric values for the two dissociation constants of carbonic acid, and the stoichiometric

TABLE I.

Variation of $pK'_{s.p.}$ $CaCO_3$ at 38° in Salt Solutions of Varying Ionic Strength.

$pK_1'' = 6.33 - 0.5\sqrt{\mu} - 0.085\mu.$

$\alpha^0_{CO_3} = 0.557 - 0.103\mu.$

Experiment No.	pCO_3, mm.	$\alpha^0_{CO_3}$	$\frac{[H_2CO_3]}{m \times 10^3 / kg.\ H_2O}$	Total $\frac{[CO_3]}{m \times 10^3 / kg.\ H_2O}$	$\frac{[HCO_3^-] + [CO_3^=]}{m \times 10^3 / kg.\ H_2O}$	$\frac{[HCO_3^-]}{[CO_3^=][H_2CO_3]}$	Log ratio.	$p_{CO}H^+$ (calculated).	pK_1'	$\frac{[HCO_3^-]}{m \times 10^3 / kg.\ H_2O}$	$\frac{[CO_3^=]}{m \times 10^3 / kg.\ H_2O}$	$\frac{[Ca^{++}]}{m \times 10^3 / kg.\ H_2O}$	pCa^{++}	$pCO_3^=$	$pK'_{s.p.}$ $CaCO_3$	μ	$\sqrt{\mu}$
1	24.3	0.556	0.794	10.30	9.51	11.98	1.079	7.35	6.274	9.49	0.0168	0.452	3.345	4.774	8.12	0.0130	0.114
1A	22.3	0.556	0.728	10.24	9.51	13.01	1.114	7.39	6.274	9.49	0.0186	0.408	3.389	4.730	8.12	0.0129	0.114
2	65.0	0.555	2.120	20.86	18.74	8.84	0.947	7.20	6.253	18.73	0.0265	0.380	3.420	4.576	8.00	0.0226	0.150
2A	44.4	0.555	1.448	20.17	18.72	12.93	1.112	7.37	6.253	18.68	0.0391	0.268	3.572	4.407	7.98	0.0225	0.150
3	61.6	0.554	2.006	30.17	28.16	14.04	1.147	7.38	6.234	28.10	0.066	0.190	3.721	4.18	7.90	0.0323	0.180
4	57.9	0.552	1.877	30.32	28.44	15.16	1.181	7.39	6.208	28.36	0.078	0.229	3.640	4.11	7.75	0.0526	0.229
4A	64.2	0.551	2.078	32.85	30.77	14.81	1.171	7.38	6.208	30.69	0.083	0.210	3.678	4.08	7.76	0.0550	0.235
5	55.1	0.550	1.781	29.98	28.20	15.84	1.200	7.39	6.189	28.11	0.085	0.238	3.623	4.07	7.69	0.0726	0.269
5A	60.1	0.549	1.938	32.51	30.57	15.77	1.198	7.39	6.189	30.48	0.092	0.233	3.633	4.04	7.67	0.0751	0.274
6	53.6	0.546	1.720	29.67	27.95	16.25	1.211	7.37	6.161	27.86	0.090	0.273	3.564	4.05	7.61	0.1029	0.321
6A	57.1	0.546	1.832	32.64	30.81	16.82	1.226	7.39	6.161	30.70	0.104	0.226	3.646	3.98	7.63	0.1055	0.325
7	51.0	0.542	1.624	29.50	27.88	17.17	1.235	7.36	6.128	27.77	0.103	0.306	3.514	3.98	7.49	0.1435	0.379
7A	53.2	0.542	1.694	32.21	30.52	18.02	1.256	7.38	6.128	30.40	0.118	0.273	3.564	3.93	7.49	0.1460	0.382
8	46.5	0.538	1.469	29.66	28.19	19.19	1.283	7.38	6.100	28.07	0.125	0.333	3.478	3.90	7.38	0.1843	0.429
8A	49.1	0.538	1.551	29.36	27.81	17.93	1.254	7.35	6.100	27.69	0.115	0.342	3.466	3.94	7.41	0.1849	0.430

values for the three dissociation constants of phosphoric acid, which corresponded with the ionic strength for which one was to calculate $pK'_{s.p.}$ The data for the carbonic acid constants and

TABLE II.

Variation of $pK'_{s.p.}$ $Ca_3(PO_4)_2$ at 38° in Salt Solutions of Varying Ionic Strength.

$$pK_1' = 2.11 - 0.5\sqrt{\mu}$$
$$pK_2' = 7.15 - 1.25\sqrt{\mu}$$
$$pK_3' = 12.66 - 2.25\sqrt{\mu}$$

Experiment No.	p_{H^+} (calculated).	Total $[PO_4]$ $\frac{M \times 10^4}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^8}{kg.\ H_2O}$	$[Ca^{++}]$ $\frac{M \times 10^8}{kg.\ H_2O}$	pCa^{++}	$pPO_4^{\equiv}$	$pK'_{s.p.}$ $Ca_3(PO_4)_2$	μ	$\sqrt{\mu}$
1	7.35	0.37	0.214	0.452	3.345	9.669	29.37	0.0130	0.114
1A	7.39	0.40	0.262	0.408	3.389	9.582	29.33	0.0129	0.114
2	7.20	1.42	0.668	0.380	3.420	9.175	28.61	0.0226	0.150
2A	7.37	1.45	1.14	0.268	3.572	8.943	28.60	0.0225	0.150
3	7.38	2.29	2.23	0.190	3.721	8.652	28.47	0.0323	0.180
4	7.39	2.51	3.35	0.229	3.640	8.475	27.87	0.0526	0.229
4A	7.38	3.02	4.12	0.210	3.678	8.385	27.80	0.0550	0.235
5	7.39	2.43	4.10	0.238	3.623	8.387	27.64	0.0726	0.269
5A	7.39	2.80	4.97	0.233	3.633	8.304	27.51	0.0751	0.274
6	7.37	2.55	5.53	0.273	3.564	8.257	27.21	0.1029	0.321
6A	7.39	3.06	7.19	0.226	3.646	8.143	27.22	0.1055	0.325
7	7.36	2.89	8.48	0.306	3.514	8.072	26.69	0.1435	0.379
7A	7.38	3.02	9.60	0.273	3.564	8.018	26.73	0.1460	0.382
8	7.38	2.59	10.77	0.333	3.478	7.968	26.37	0.1843	0.429
8A	7.35	2.70	10.43	0.342	3.466	7.982	26.36	0.1849	0.430

those for phosphoric acid were obtained from various sources described in the preceding papers in sections devoted to the subject.

Upon plotting the experimental points for $pK'_{s.p.}$ against the square root of the ionic strength it was seen that the curves for carbonate and phosphate deviated slightly from a straight line. The lowest ionic strength in these experiments being a little over

0.01 ($\sqrt{\mu} = 0.114$), a graphical extrapolation to zero ionic strength seemed a questionable procedure. In order, however, to check it up with theory, the following treatment was employed to locate the activity solubility product, $pK'_{s.p.}$, and at the same time to determine the best theoretical curve, running from the solubility product at zero ionic strength, through the stoichiometric solubility products at increasing salt concentrations.

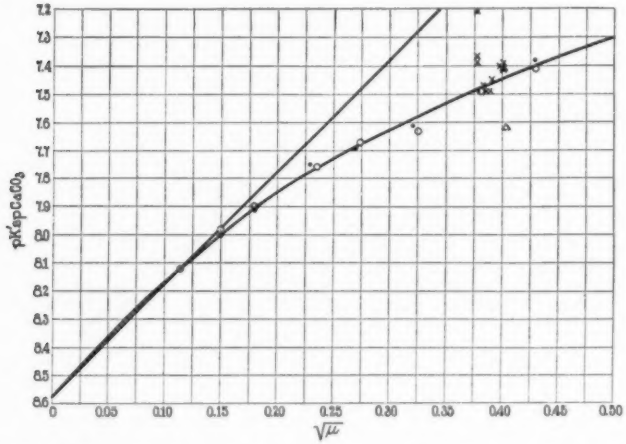

FIG. 1. The solubility of $CaCO_3$ in salt solutions of varying ionic strength saturated with $CaCO_3$ and $Ca_3(PO_4)_2$ at 38°. Values of $pK'_{s.p.}CaCO_3$ are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The curved line has the equation $pK'_{s.p.}CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.85\sqrt{\mu}}$.

According to Hückel, the activity coefficient of strong electrolytes varies with the ionic strength in accordance with a formula which has the general form $-\log \gamma_{salt} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}} + C\mu$ (see equations (20) and (21), Paper I) where B, A, and C are constants depending, among other factors, upon the electronic environment, secondary electrical effects such as "salting out" or hydration, the average size of the ions, and the change in dielectric constant of the solvent with salt. In the case of such

slightly soluble salts as $CaCO_3$ and $Ca_3(PO_4)_2$, one may assume complete dissociation and apply directly the theory of strong electrolytes as presented in the section on theory. In the case of weak electrolytes, which obey the classical law of mass action, the ions must of necessity be subject to the same influences as those of strong electrolytes.

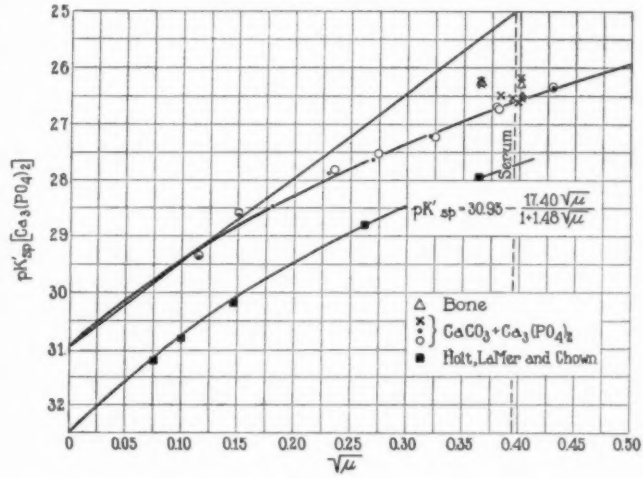

FIG. 2. The solubility of $Ca_3(PO_4)_2$ in salt solutions of varying ionic strength saturated with $CaCO_3$ and $Ca_3(PO_4)_2$ at 38°. Values of $pK'_{s.p.}$ $Ca_3(PO_4)_2$ are plotted as ordinates and $\sqrt{\mu}$ as abscissæ. The curved line has the equation $pK'_{s.p.}Ca_3(PO_4)_2 = 30.95 - \frac{17.40\sqrt{\mu}}{1 + 1.48\sqrt{\mu}}$.

Calculation of $pK'_{s.p.}CaCO_3$.—According to the equations of Paper I,

$$(6) \qquad a_{Ca^{++}} \times a_{CO_3^{=}} = K_{s.p.}\ CaCO_3$$

$$(7) \qquad \gamma_{Ca^{++}}[Ca^{++}] \times \gamma_{CO_3^{=}}[CO_3^{=}] = K_{s.p.}\ CaCO_3$$

$$(8) \qquad [Ca^{++}][CO_3^{=}] = \frac{K_{s.p.}\ CaCO_3}{\gamma_{Ca^{++}} \times \gamma_{CO_3^{=}}} = K'_{s.p.}\ CaCO_3$$

or $pK_{s.p.}\ CaCO_3 = pK'_{s.p.}\ CaCO_3 + p\ \gamma_{Ca^{++}} + p\ \gamma_{CO_3^{=}}$

But since by definition, the activity coefficient of a salt is the geometrical mean of the activity coefficients of its ions,

$$(9) \qquad \frac{p\gamma_{Ca^{++}} + p\gamma_{CO_3^{=}}}{2} = p\gamma_{CaCO_3}$$

$$(10) \qquad \text{or } pK_{s.p.}\ CaCO_3 - pK'_{s.p.}\ CaCO_3 = 2\ p\gamma_{CaCO_3}$$

$$(11) \qquad \Delta pK'_{s.p.}\ CaCO_3 = 2\ p\gamma_{CaCO_3}$$

According to Brönsted and La Mer (1924)

$$\frac{\Delta pK'_{s.p.}}{\nu} = -\log_{10}\gamma_{salt} = \alpha' z_1 z_2 \sqrt{\mu}$$

where ν = number of ions of the salt, α' is a universal constant, and z_1 and z_2 are the valences of the ions. Hence

$$(12) \qquad \Delta\ pK'_{s.p.}\ CaCO_3 = 2p\gamma_{CaCO_3} = 4\sqrt{\mu}$$

Their expression holds for highly dilute solutions only, but by the addition of another term $0.87\beta c$, it may be corrected so that it is valid to a concentration of $\mu = 0.2$. For higher concentrations, the formula of Hückel (1925) is better suited, and we have accordingly adopted it, omitting the correction factor $C\mu$.

Drawing graphically an approximately average curve through the sixteen points for $pK'_{s.p.}\ CaCO_3$ and extrapolating to zero ionic strength, $pK'_{s.p.}\ CaCO_3$ was found to be approximately 8.6. Since equation (12) holds for dilute solutions, it was applied in its simplest form to the points of lowest ionic strength, namely 1, 1A, 2, 2A. These gave the average value 8.58 for $pK_{s.p.}\ CaCO_3$, which agreed with that found in experiments already described with only $CaCO_3$ as the saturating body. This figure was then used as the basis of further calculations to obtain the constants of equation (18). Of course, the two methods of extrapolation, although they agree quite well, afford no assurance that the solubility product at infinite dilution is any more accurate than to about 0.1. However, the value found, arbitrary though it may be, serves its purpose. It furnishes the basis for calculating the expression representative of the empirical points found, as placed on a theoretical curve.

According to equations (21), Paper I, and (11),

$$(13) \qquad p\gamma_{CaCO_3} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}} = \frac{\Delta\ pK'_{s.p.}\ CaCO_3}{2}$$

By inversion and separation into different terms,

$$(14) \qquad \frac{2}{\Delta\ pK'_{s.p.}\ CaCO_3} = \frac{1 + A\sqrt{\mu}}{B\sqrt{\mu}} = \frac{1}{B\sqrt{\mu}} + \frac{A}{B}$$

Thus, when plotting $\frac{2}{\Delta\ pK'_{s.p.}\ CaCO_3}$ against $\frac{1}{\sqrt{\mu}}$, a line can be drawn which determines the empirical constants B and A, the slope being $\frac{1}{B}$, the intercept $\frac{A}{B}$. Thus

$$\frac{2}{\Delta\ pK'_{s.p.}\ CaCO_3} = \frac{1 + 1.85\sqrt{\mu}}{2.47\sqrt{\mu}} \tag{15}$$

or

$$p\gamma_{CaCO_3} = \frac{2.47\sqrt{\mu}}{1 + 1.85\sqrt{\mu}} = \frac{\Delta\ pK'_{s.p.}\ CaCO_3}{2} \tag{16}$$

or

$$2p\gamma_{CaCO_3} = \frac{4.94\sqrt{\mu}}{1 + 1.85\sqrt{\mu}} = \Delta pK'_{s.p.}\ CaCO_3 = p\gamma_{Ca^{++}} + p\gamma_{CO_3^{=}} \tag{17}$$

Hence, by equations (10) and (17), the equation for the theoretical line through the experimental points will be

$$pK'_{s.p.}\ CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1 + 1.85\sqrt{\mu}} \tag{18}$$

Table III shows how those points fit the calculated curve. Of course, they must fit fairly well, since the constants B and A were derived by using them in calculations. However, the possibility of working in such a manner with the results recorded may be regarded as an index of the accuracy of the experimental work.

We cannot compare our estimations directly with those of Johnston because of the fact that our measurements were carried out at 38°, whereas his were made at 16°. From the work of Leather and Sen (1909), Johnston (1915) calculated that the solubility product of calcium carbonate at 40° was 0.5×10^{-8}, or $pK'_{s.p.}CaCO_3 = 8.30$. The ionic strength of the solution was approximately 0.005 which corresponds to the value 8.29 for $pK'_{s.p.}CaCO_3$ on Fig. 1. This is cited to show that the order of magnitude of our values is not inconsistent with what has been found in past studies on $CaCO_3$ solubility.

Attention may be called here to the difference in the two curves

for $pK'_{s.p.}CaCO_3$ obtained when salt solutions are saturated with $CaCO_3$ in the presence and absence of $Ca_3(PO_4)_2$. By equation (63), Paper I, when $\mu = 0.16$, $pK'_{s.p.}CaCO_3 = 7.38$, while equation (18) gives $pK'_{s.p.}CaCO_3 = 7.44$. As can be seen for equations (18) and (63), Paper I, the effect is marked only at the higher concentrations and is apparently real, for the averages of

TABLE III.

Comparison of Observed and Calculated $pK'_{s.p.}CaCO_3$ at 38°, at Various Ionic Strengths.

Experiment No.	$\sqrt{\mu}$	$pK'_{s.p.}$ calculated.	$pK'_{s.p.}$ observed.	$pK'_{s.p.}$ deviation.
1	0.114	8.12	8.12	0.00
1A	0.114	8.12	8.12	0.00
2	0.150	8.00	8.00	0.00
2A	0.150	8.00	7.98	−0.02
3	0.180	7.91	7.90	−0.01
4	0.229	7.78	7.75	−0.03
4A	0.235	7.77	7.76	−0.01
5	0.269	7.69	7.69	0.00
5A	0.274	7.68	7.67	−0.01
6	0.321	7.58	7.61	+0.03
6A	0.325	7.58	7.63	+0.05
7	0.379	7.48	7.49	+0.01
7A	0.382	7.48	7.49	+0.01
8	0.429	7.40	7.38	−0.02
8A	0.430	7.40	7.41	+0.01

the several different determinations at this region under the two conditions also give values which are distinctly different. Apparently, the presence of $PO_4^{\equiv}$ ion, of unsymmetrical valence type with respect to Ca^{++} ion, exerts some secondary salt effect. As a consequence of the theory of strong electrolytes from the standpoint of electronic forces between ions and their surrounding environment, this discrepancy might be expected. It is unknown,

however, whether the slight amount of phosphate in these solutions could account for the differences in the two curves.

Calculation of $pK'_{s.p.}$ $Ca_3(PO_4)_2$.—This development is analogous to the one for calcium carbonate:

$$(\alpha_{Ca^{++}})^3 \times (\alpha_{PO_4^{\equiv}})^2 = K_{s.p.}\ Ca_3(PO_4)_2 \tag{2}$$

$$(\gamma_{Ca^{++}})^3\,[Ca^{++}]^3 \times (\gamma_{PO_4^{\equiv}})^2\,[PO_4^{\equiv}]^2 = K_{s.p.}\ Ca_3(PO_4)_2 \tag{19}$$

$$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = \frac{K_{s.p.}\ Ca_3(PO_4)_2}{(\gamma_{Ca^{++}})^3\,(\gamma_{PO_4^{\equiv}})^2} = K'_{s.p.}\ Ca_3(PO_4)_2 \tag{20}$$

or, transforming to logarithmic form,

$$pK_{s.p.}\ Ca_3(PO_4)_2 = pK'_{s.p.}\ Ca_3(PO_4)_2 + 3\ p\gamma_{Ca^{++}} + 2\ p\gamma_{PO_4^{\equiv}} \tag{21}$$

$$\frac{3\ p\gamma_{Ca^{++}} + 2\ p\gamma_{PO_4^{\equiv}}}{5} = p\gamma_{Ca_3(PO_4)_2} \tag{22}$$

Hence,

$$pK_{s.p.}\ Ca_3(PO_4)_2 - pK'_{s.p.}\ Ca_3(PO_4)_2 = 5\ p\gamma_{Ca_3(PO_4)_2} \tag{23}$$

$$\Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2 = 5\ p\gamma_{Ca_3(PO_4)_2} \tag{24}$$

By equation (19), Paper I,

$$\Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2 = 5\ p\gamma_{Ca_3(PO_4)_2} = 15\sqrt{\mu} \tag{25}$$

for dilute solutions. Extrapolating back graphically, and with the aid of equation (25) from points of lowest ionic strength 1, 1A, 2, and 2A, the activity solubility product at zero ionic strength was found to be 30.95. Such an extrapolation involves an error in $pK_{s.p.}\ Ca_3(PO_4)_2$ of probably 0.3.

Although Hückel's equation has not been applied to salts of unsymmetrical higher valence type, in the absence of any other method that would yield a better expression for the variation of $pK'_{s.p.}\ Ca_3(PO_4)_2$ with ionic strength, it was decided to employ such an equation to fit the data, as had already been done for calcium carbonate. With this reservation, the calculations were performed as before.

$$p\gamma_{Ca_3(PO_4)_2} = \frac{B\sqrt{\mu}}{1 + A\sqrt{\mu}} = \frac{\Delta pK'_{s.p.}\ Ca_3(PO_4)_2}{5} \tag{26}$$

$$\frac{5}{\Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2} = \frac{1 + A\sqrt{\mu}}{B\sqrt{\mu}} = \frac{1}{B\sqrt{\mu}} + \frac{A}{B} \tag{27}$$

Plotting $\frac{5}{\Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2}$ against $\frac{1}{\sqrt{\mu}}$ as before,

B = 3.48 and A = 1.48. Accordingly, equation (26) becomes

$$(28) \qquad p\gamma_{Ca_3(PO_4)_2} = \frac{3.48\sqrt{\mu}}{1 + 1.48\sqrt{\mu}} = \frac{\Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2}{5}$$

TABLE IV.

Comparison of Observed and Calculated $pK'_{s.p.}$ $Ca_3(PO_4)_2$ at 38°, at Various Ionic Strengths.

Experiment No.	$\sqrt{\mu}$	$pK'_{s.p.}$ calculated.	$pK'_{s.p.}$ observed.	$pK'_{s.p.}$ deviation.
1	0.114	29.25	29.37	+0.12
1A	0.114	29.25	29.33	+0.08
2	0.150	28.82	28.61	−0.21
2A	0.150	28.82	28.60	−0.22
3	0.180	28.48	28.47	−0.01
4	0.229	27.97	27.87	−0.10
4A	0.235	27.92	27.80	−0.12
5	0.269	27.60	27.64	+0.04
5A	0.274	27.56	27.51	−0.05
6	0.321	27.16	27.21	+0.05
6A	0.325	27.13	27.22	+0.09
7	0.379	26.73	26.69	−0.04
7A	0.382	26.70	26.73	+0.03
8	0.429	26.39	26.37	−0.02
8A	0.430	26.38	26.36	−0.02

or

$$(29) \quad 5\ p\gamma_{Ca_3(PO_4)_2} = \frac{17.40\sqrt{\mu}}{1 + 1.48\sqrt{\mu}} = \Delta\ pK'_{s.p.}\ Ca_3(PO_4)_2 = 3\ p\gamma_{Ca^{++}} + 2\ p\gamma_{PO_4^{\equiv}}$$

Hence, by equations (29) and (21) the equation for the theoretical line through the experimental points will be

$$(30) \qquad pK'_{s.p.}\ Ca_3(PO_4)_2 = 30.95 - \frac{17.40\sqrt{\mu}}{1 + 1.48\sqrt{\mu}}$$

Table IV shows the difference between actual experimental points and the calculated curve.

At this point, it may be interesting to compare the foregoing results with the curve for $pK'_{s.p.}Ca_3(PO_4)_2$ obtained by Holt, La Mer, and Chown ((1925, *a*) p. 544), in which the ionic strength was varied by means of KCl and NaCl. They apparently did not distinguish between K, the dissociation constant at infinite dilution, and K', the stoichiometric or apparent dissociation constant of an acid. Consequently, it may be expected that the use of the three dissociation constants of phosphoric acid at the same value throughout would give a curve which would be differ-

TABLE V.

Variation in $pK'_{s.p.}$ $Ca_3(PO_4)_2$ at 38° with Varying Ionic Strength.

Recalculated from Holt, La Mer, and Chown (1925, *a*), Table V, p. 538. $Ca(OH)_2+H_3PO_4$, shaking 8 days.

Experiment No.	pH+	$[Ca^{++}]$ $m \times 10^3$ / kg. H_2O	Total $[PO_4]$ $m \times 10^3$ / kg. H_2O	$[H_2PO_4^-]$ $m \times 10^3$ / kg. H_2O	$[HPO_4^=]$ $m \times 10^3$ / kg. H_2O	$[PO_4^{\equiv}]$ $m \times 10^{12}$ / kg. H_2O	pCa++	$pPO_4^{\equiv}$	$pK'_{s.p.}$ $Ca_3(PO_4)_2$	μ	$\sqrt{\mu}$
1	5.21	1.96	3.71	3.66	0.053	2.84	2.707	11.547	31.22	0.0058	0.076
2	5.27	1.98	3.63	3.57	0.064	4.38	2.703	11.358	30.82	0.0098	0.099
3	5.33	2.00	3.58	3.49	0.084	8.99	2.699	11.046	30.19	0.0216	0.147
4	5.46	2.07	3.54	3.39	0.155	41.62	2.684	10.381	28.81	0.0690	0.263
5	5.51	2.12	3.54	3.33	0.214	98.2	2.673	10.007	28.03	0.1324	0.364
6	5.52	2.22	3.59	3.37	0.223	104	2.653	9.983	27.93	0.1326	0.364
7	5.53	3.11	4.04	3.65	0.391	428	2.507	9.368	26.26	0.2618	0.512

ent from one that would be obtained by the use of constants, which have been corrected for the known increase which takes place with increasing concentration. Accordingly, their values have been recalculated using the constants as given in the preceding paper, and placing their concentrations on a molality instead of a molarity basis. This was done by using a water content which varied with the concentration according to the approximate formula obtained at room temperature at this laboratory; namely,

$$(996 - 30\,\mu) = \text{gm. of } H_2O \text{ per liter.} \tag{31}$$

The results are shown in Table V and Fig. 2.

It is at once evident that the two curves differ considerably in

the value for $pK'_{s.p.}Ca_3(PO_4)_2$ at zero ionic strength, their curve giving on extrapolation, $pK_{s.p.}\ Ca_3(PO_4)_2 = 32.50$ as compared with our value of 30.95.

Inasmuch as the two sets of data were obtained from two different systems, it is impossible to tell whether technique or some other factor is the source of the discrepancy. The experiments recorded in these papers were done in the presence of solid $CaCO_3$ and $Ca_3(PO_4)_2$; theirs were obtained by adding $Ca(OH)_2$ to H_3PO_4 and shaking for 8 days to permit complete precipitation. In these particular systems, when the solid is present initially one might expect to attain equilibrium sooner than when waiting for the solid to precipitate. Their reaction ($p\alpha_{H^+} = 5.21$ to 5.53) resulted in a much smaller concentration of $PO_4^{\equiv}$ ion and a correspondingly greater concentration of $HPO_4^{=}$, $H_2PO_4^{-}$, and Ca^{++} ions. In view of their demonstration of the specific effect of excess $H_2PO_4^{-}$ ion on $pK'_{s.p.}Ca_3(PO_4)_2$, it is difficult to understand why that ion did not exert a similar effect in these solutions. Even in the presence of NaCl and KCl, the tendency to increase the stoichiometric solubility product with increasing concentration, should have been much more than it was. Their $p\alpha_{H^+}$ values, on the basis of N/10 HCl = 1.04 would make $pK'_{s.p.}Ca_3(PO_4)_2$ even greater by 0.08 or 0.09. Inasmuch as it is not known whether there was any correction made for diffusion potential, their data have been recalculated at their original $p\alpha_{H^+}$ values.

Activity Coefficients of $CaCO_3$, $Ca_3(PO_4)_2$, and Ca^{++} Ion in Salt Solutions of Varying Ionic Strength.—The experiments on the variation in stoichiometric solubility product of $CaCO_3$ and $Ca_3(PO_4)_2$ with varying salt concentration, enable one to calculate the activity coefficient of the salts. By utilizing the activity coefficients of the $CO_3^{=}$ ion and the $PO_4^{\equiv}$ ion given in the section on constants and equations, one may calculate the activity coefficient of Ca^{++} ion and its variation with ionic strength.

By definition, the activity coefficient of a salt is the geometrical mean of the activity coefficients of its ions. Thus

$$(\gamma_{Ca^{++}})^3 \times (\gamma_{PO_4^{\equiv}})^2 = (\gamma_{Ca_3(PO_4)_2})^5 \text{ or}$$

$$3\ p\gamma_{Ca^{++}} + 2\ p\gamma_{PO_4^{\equiv}} = 5\ p\gamma_{Ca_3(PO_4)_2} \tag{22}$$

Therefore, it will be necessary to evaluate the activity coefficient variation with ionic strength, for the anion of the particular calcium salt being studied. According to Hastings and Sendroy (1925)

(32) $$p\gamma_{CO_3^=} = 1.6\sqrt{\mu}$$

(9) $$2\ p\gamma_{CaCO_3} = p\gamma_{Ca^{++}} + p\gamma_{CO_3^=}$$

By substituting the value of $2\ p\gamma_{CaCO_3}$ from equation (9)

(33) $$p\gamma_{Ca^{++}} = 2\ p\gamma_{CaCO_3} - p\gamma_{CO_3^=} = \frac{4.94\sqrt{\mu}}{1 + 1.85\sqrt{\mu}} - 1.6\sqrt{\mu}$$

or

(34) $$p\gamma_{Ca^{++}} = \left(\frac{4.94}{1 + 1.85\sqrt{\mu}} - 1.6\right)\sqrt{\mu}$$

In the same way, using $p\gamma_{PO_4^{\equiv}}$ as derived in Paper II, we have

(28) $$p\gamma_{PO_4^{\equiv}} = 4.0\sqrt{\mu}$$

(22) $$5\ p\gamma_{Ca_3(PO_4)_2} = 3\ p\gamma_{Ca^{++}} + 2\ p\gamma_{PO_4^{\equiv}}$$

(35) $$p\gamma_{Ca^{++}} = \tfrac{1}{3}\ p\gamma_{Ca_3(PO_4)_2} - \tfrac{2}{3}\ p\gamma_{PO_4^{\equiv}}$$

$$= \tfrac{1}{3}\left(\frac{3.48\sqrt{\mu}}{1 + 1.48\sqrt{\mu}}\right) - \tfrac{2}{3}\ (4.0\sqrt{\mu})$$

$$= \frac{5.80\sqrt{\mu}}{1 + 1.48\sqrt{\mu}} - 2.67\sqrt{\mu}$$

or

(36) $$p\gamma_{Ca^{++}} = \left(\frac{5.80}{1 + 1.48\sqrt{\mu}} - 2.67\right)\sqrt{\mu}$$

Table VI and Fig. 3 represent the values for the activity coefficients of $CaCO_3$ and $Ca_3(PO_4)_2$ varying with ionic strength, calculated according to the equations of the preceding paragraphs. Column 5 gives Ca^{++} ion activity coefficient values from the $CaCO_3$ solubility data, while those of Column 9 are derived from

the corresponding data on $Ca_3(PO_4)_2$. Examination of the carbonate data reveals the fact that even at the lowest concentrations, the activity coefficients of $CO_3^=$ ion and Ca^{++} ion are different. This is in accord with what little evidence has so far been obtained on individual ion activities. The activity coeffi-

TABLE VI.

Variation with Ionic Strength of the Activity Coefficients of $CaCO_3$, $Ca_3(PO_4)_2$, and Ca^{++} Ion at 38°.

Experiment No. (1)	$p\gamma_{CaCO_3}$ (2)	γ_{CaCO_3} (3)	$p\gamma_{Ca^{++}}$ (4)	$\gamma_{Ca^{++}}$ (5)	$p\gamma_{Ca_3(PO_4)_2}$ (6)	$\gamma_{Ca_3(PO_4)_2}$ (7)	$p\gamma_{Ca^{++}}$ (8)	$\gamma_{Ca^{++}}$ (9)	μ (10)	$\sqrt{\mu}$ (11)
1	0.230	0.588	0.278	0.527	0.340	0.457	0.263	0.546	0.0130	0.114
1A	0.230	0.588	0.278	0.527	0.340	0.457	0.263	0.546	0.0129	0.114
2	0.290	0.512	0.340	0.457	0.426	0.375	0.307	0.493	0.0226	0.150
2A	0.290	0.512	0.340	0.457	0.426	0.375	0.307	0.493	0.0225	0.150
3	0.335	0.462	0.382	0.415	0.494	0.320	0.343	0.454	0.0323	0.180
4	0.400	0.398	0.434	0.368	0.596	0.253	0.382	0.415	0.0526	0.229
4A	0.405	0.402	0.434	0.368	0.606	0.248	0.383	0.414	0.0550	0.235
5	0.445	0.359	0.460	0.347	0.670	0.214	0.399	0.399	0.0726	0.269
5A	0.450	0.354	0.462	0.345	0.678	0.210	0.398	0.400	0.0751	0.274
6	0.500	0.316	0.486	0.326	0.758	0.175	0.407	0.392	0.1029	0.321
6A	0.500	0.316	0.480	0.331	0.764	0.172	0.406	0.392	0.1055	0.325
7	0.550	0.282	0.494	0.320	0.844	0.143	0.395	0.403	0.1435	0.379
7A	0.550	0.282	0.489	0.324	0.850	0.141	0.397	0.401	0.1460	0.382
8	0.590	0.257	0.494	0.320	0.912	0.122	0.374	0.422	0.1843	0.429
8A	0.590	0.257	0.492	0.322	0.914	0.122	0.375	0.421	0.1849	0.430

cients of the cation and anion in solutions of a difficultly soluble salt, do not necessarily have to be equal, even when cation and anion are of similar valence type. With increasing ionic strength, one would expect such differences to increase, as they do in these experiments.

Lewis and Randall (p. 362) have collected into one table the activity coefficients of typical electrolytes as established by freezing point data. We reproduce here their values for the molality of 0.01 followed by our own values for $CaCO_3$ and $Ca_3(PO_4)_2$ calculated at the same concentration.

HCl (25°)	LiCl (25°) NaCl (25°) KCl (25°)	KOH (25°)	KNO_3	$AgNO_3$	KIO_3 $NaIO_3$	$BaCl_2$
0.924	0.922	0.92	0.916	0.902	0.882	0.716
$CdCl_2$ (25°)	K_2SO_4	H_2SO_4 (25°)	$La(NO_3)_3$. $K_3Fe(CN)_6$ (p. 344)	$MgSO_4$ $CdSO_4$ $CuSO_4$	$CaCO_3$ (38°)	$Ca_3(PO_4)_2$ (38°)
0.532	0.687	0.617	0.571	0.404	0.436	0.139

Here, apparently, the activity coefficient decreases as the complexity of the salt grows with increasing number of ions in the molecule, increasing valence of the ions, and dissimilarity in pairs of ions constituting the salt. The activity coefficients of $CaCO_3$ and $Ca_3(PO_4)_2$ at $c = 0.01$, were calculated according to equations (34) and (36) respectively.

The values for $CaCO_3$ and $Ca_3(PO_4)_2$ $pK_{s.p.}$ are those which would be given by our equations on the basis of μ in NaCl solutions. If only Ca^{++} and $CO_3^{=}$ ions or Ca^{++} and $PO_4^{\equiv}$ ions contributed to the entire ionic strength at these molal concentrations, *i.e.* if we could possibly have only a pure solution of $CaCO_3$ and $Ca_3(PO_4)_2$, perhaps these values would be different. As it is, not having such data, it has been necessary to resort to the calculation indicated above. The rule given by Lewis and Randall that the activity coefficient of a given electrolyte is the same in all solutions of the same ionic strength, is a limiting law and does not hold rigidly with increasing concentration and increasing complexity of the salt. As has been noted before, even at the same ionic strength, at a concentration such as these given here, the nature of the ions in solution has marked specific effects on the thermodynamic properties of such a solution. Bearing in mind that the activity coefficient of such a salt as $Ca_3(PO_4)_2$ may be quite different in a solution where only Ca^{++} and $PO_4^{\equiv}$ ions are present from one in which NaCl is predominant, we will note the relative activity coefficients of a few salts at $\mu = 0.04$. The

sulfate value is obtained from the same source as those above, the $CaCO_3$ and $Ca_3(PO_4)_2$ being calculated according to equations (34) and (36) respectively.

$MgSO_4$
$CdSO_4$
$CuSO_4$
$CaSO_4$ (p. 376) } 0.404; $CaCO_3$ (38°), 0.436; $Ca_3(PO_4)_2$ (38°), 0.289

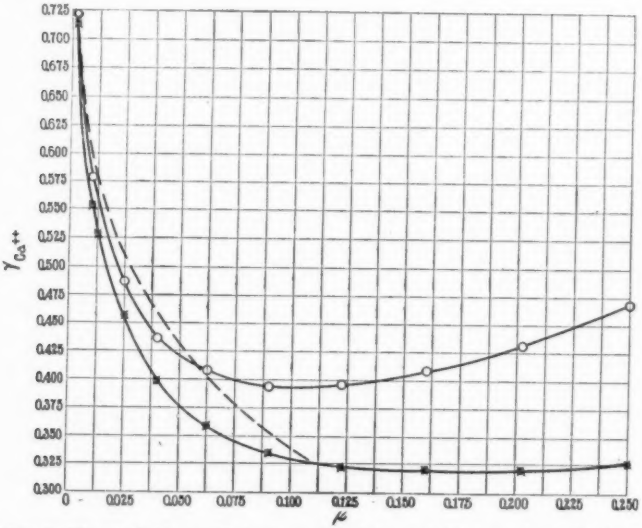

FIG. 3. The activity coefficient, γ, of calcium plotted against the ionic strength μ, calculated from $CaCO_3$ solubility data and from $Ca_3(PO_4)_2$ solubility data. The former are designated by ○, the latter by ⊗. The broken line is plotted from data given in Lewis and Randall's Thermodynamics, p. 382, for divalent ions.

The low value for $Ca_3(PO_4)_2$ may be due to the errors involved either in calculating for μ as we have noted above, in using doubtful dissociation constant values for phosphoric acid, or to a number of other unknown factors such as degree of hydration of the ions.

Considering the difficulties involved, it is not surprising to find that the activity coefficients of Ca^{++} ion, calculated from the solubility data of the two salts, are different. However, inasmuch

as both values have been obtained on exactly the same solutions, and the Ca^{++} ion activity at each concentration can have but one value, we must conclude that the discrepancy is a result of one of the factors mentioned above. Probably most of the difference represents the result of the combined error that may be involved in using the particular dissociation constants for carbonic acid and phosphoric acid which have been adopted for the calculations.

Fig. 3 indicates the variation of the activity coefficient, $\gamma_{Ca^{++}}$, with ionic strength. We have also plotted the few points of Lewis and Randall (p. 382) of average values for several bivalent cations, Ca^{++} included, calculated from the activity coefficient of the corresponding halides and that of Cl^- ion. These check our experimental results up to $\mu = 0.1$. Our results, however, show that the decrease in value of γ ceases at the low ionic strength of 0.1 in one case and of 0.2 in that of the other. This is quite unlike the behavior of the activity coefficient of Ca^{++} as determined from $CaSO_4$ data. Results such as these may be due to difference in electronic environment or inaccuracies in the use of the acid activity coefficients with varying μ.

To quote from Harned (1924), "It would appear that the activity coefficient of an ion at constant temperature is a function of its concentration, its valence, its dimensions, its complexity and its interaction with the other ions in the solution containing it. At higher concentrations, the effect of hydration becomes prominent as a factor which probably causes the thermodynamic behaviors of the ions to differ." It would seem that the difficulties attendant to the study of the activity of individual ions are enormous, and that many of the ideas on the subject are to be regarded as no more than tentative, awaiting further extension of our knowledge before further refinements in accuracy are possible. It is with this view-point that the material related to calcium ion and salt activities has been presented.

Solubility of $CaCO_3$ and $Ca_3(PO_4)_2$ in Salt Solutions under Various Conditions.—In order to obtain light on the question of the precipitation and solution of calcium salts in biological fluids, it was considered desirable to study the effect of the various conditions under which equilibrium with the two saturating salts $CaCO_3$ and $Ca_3(PO_4)_2$ could be attained. The effect of varying ionic strength has been described. The time limit of equilibrium

between solid and liquid phase, the possible variation in results with differences in the initial composition of the salt solutions, and incidentally the effect, if any, of differences in $p\alpha_{H^+}$ were investigated.

In order to simulate as nearly as possible the composition of serum without its protein, balanced salt solutions already described were used. It was found that, contrary to expectation, such an artificial edema fluid gave no evidence of specific effect of the other ions present, but behaved as if it were entirely a NaCl solution of the same ionic strength.

One of the first considerations was the question of the effect of such a solution containing the various ions on the activity coefficient of the bicarbonate ion. This had been determined previously only in the presence of NaCl and $NaHCO_3$. In view of the marked specific effect of higher valence type ions on the electronic properties of solutions, pK_1'' of carbonic acid was determined in four of the saturation systems, Nos. 10, 11, 14, and 15, and in another group in which artificial edema fluid was used without saturation with solid or addition of Ca^{++} ion. Calculations were made according to equation (50). The results are given in Table VII. It was found that the average value of the saturation system determinations gave $pK_1'' = 6.135$ and the other three gave 6.154. Hence it was felt that the use of $pK_1'' = 6.13$ on the basis of the determinations in NaCl solutions previously made by Hastings and Sendroy, was justified in the case of these solutions. Any error in using the wrong value of pK_1'' would be of no greater magnitude than the error involved in calculation with a similar discrepancy in $p\alpha_{H^+}$. Further, the $p\alpha_{H^+}$ of "30," "40," and "50" was determined both electrometrically and colorimetrically as indicated in the table, with practically no difference in results. This served as a check on the maintenance of the value of the apparent dissociation constant of phenol red which had previously been determined for the bicolor standards against M/15 phosphate solution. In view of these results, it was considered correct to apply all data on sodium chloride solutions of the same ionic strength to the artificial edema fluid used in these experiments.

Table VIII gives a general summary of the results of this group of experiments. The experiments have been separated into four

groups, according to the initial composition, and will be treated separately in that manner in the analysis which follows.

Group I. Experiments 9 and 14.—In this group calcium and phosphate were initially absent from the solutions. In order to satisfy the stoichiometric solubility product of both $CaCO_3$ and $Ca_3(PO_4)_2$, the two salts dissolved, furnishing Ca^{++}, $CO_3^{=}$, and $PO_4^{\equiv}$ ions. Any excess of Ca^{++} over the carbonate solubility product would cause reprecipitation as the carbonate. Inasmuch as in this case the amount of $Ca_3(PO_4)_2$ dissolved furnished just

TABLE VII.

Determination of pK_1'' H_2CO_3 in Artificial Edema Fluid.

Experiment No.	pCO_2, mm.	$\alpha^0_{CO_2}$	$[H_2CO_3]$ $M \times 10^5$ / kg. H_2O	Total $[CO_2]$ $M \times 10^5$ / kg. H_2O	$[HCO_3^-]$ $M \times 10^5$ / kg. H_2O	pcpH+	$\frac{[HCO_3^-]}{[H_2CO_3]} = R$	log R	pK_1''	μ	$\sqrt{\mu}$
"30"	30.9	0.541	0.982	30.97	29.83	7.633e	30.39	1.483	6.150	0.1503	0.387
"40"	38.5	0.541	1.223	31.59	30.25	7.541e	24.72	1.393	6.148	0.1506	0.388
"50"	47.8	0.541	1.519	31.79	30.18	7.466e	19.87	1.298	6.164	0.1506	0.388
Average									6.154		
10	38.6	0.541	1.23	28.53	27.16	7.47c	22.08	1.34	6.13	0.1478	0.385
11	39.1	0.541	1.24	29.42	27.98	7.46c	22.57	1.35	6.11	0.1436	0.379
14	41.5	0.541	1.32	31.35	29.94	7.51c	22.69	1.36	6.15	0.1497	0.387
15	36.3	0.541	1.15	28.24	27.00	7.52c	23.48	1.37	6.15	0.1423	0.377
Average									6.135		

about the right amount of Ca^{++} ion to satisfy both solubility products, there was but little precipitation of the calcium as carbonate. There was no difference in $pK'_{s.p.}CaCO_3$, but some in $pK'_{s.p.}Ca_3(PO_4)_2$, with difference in time of saturation from 1 day to 8 days.

Group II. Experiment 8.—The initial Ca^{++} ion concentration was quite high, $[HCO_3^-]$ and $[CO_3^{=}]$ normal, but no phosphate was initially in solution. At first the high $[Ca^{++}]$ caused precipitation of the bicarbonate as $CaCO_3$, and, as the Ca^{++} ion concentration decreased, the driving force of the reaction decreased. In the

TABLE VIII.

Solubility of $CaCO_3$ and $Ca_3(PO_4)_2$ in Salt Solutions Saturated at 38° with Both Solids.

Experiment No.	Saturation time.	Initial concentrations.			Final concentrations.							pK'$_{s.p.}$ $CaCO_3$	pK'$_{s.p.}$ $Ca_3(PO_4)_2$	$\sqrt{\mu}$	Reaction.
		$[HCO_3^-]$ M × 10³ / kg. H_2O	Total $[PO_4]$ M × 10³ / kg. H_2O	$[Ca^{++}]$ M × 10³ / kg. H_2O	Total $[CO_3]$ M × 10³ / kg. H_2O	$[HCO_3^-]$ M × 10³ / kg. H_2O	$[CO_3^=]$ M × 10³ / kg. H_2O	Total $[PO_4]$ M × 10³ / kg. H_2O	$[PO_4^{\equiv}]$ M × 10³ / kg. H_2O	$[Ca^{++}]$ M × 10³ / kg. H_2O	p_cH^+			
	hrs.														
9	20	30.27	None.	None.	31.04	29.43	0.129	0.275	1.05	0.31	7.43c	7.40	26.49	0.383	CO_3 ppt. PO_4 diss.
14	8 days.	30.27	"	"	31.35	29.92	0.157	0.133	0.625	0 26	7.51c	7.39	27.17	0.387	" "
8	20	30.27	None.	7.76	19.20	17.64	0.045	0.038	0.078	1.33	7.20c	7.23	26.86	0.378	CO_3 ppt. PO_4 diss.
16	44	30.27	2.42	None.	33.62	32.08	0.165	1.06	4.86	0.25	7.50c	7.38	25.42	0.378	CO_3 diss. PO_4 ppt.
24	7 days.	30.27	2.42	"	33.67	32.31	0.195	0.93	5.11	0.23	7.57c	7.35	25.50	0.380	" "
5C	17	30.27	0.24	0.50	30.07	28.74	0.154	0.25	1.21	0.26	7.52c	7.40	26.61	0.398	CO_3 ppt. PO_4 ppt.
B	18	30.27	1.21	2.52	27.53	26.53	0.188	0.332	2.18	0.228	7.64c	7.37	26.24	0.365	" "
D	18	29.67	1.19	2.47	26.93	25.95	0.180	0.324	2.08	0.228	7.63c	7.39	26.28	0.365	" "
7′	18	30.27	0.71	1.51	29.50	27.77	0.103	0.289	0.848	0.306	7.36g	7.49	26.69	0.379	" "
7A′	18	32.80	0.71	1.51	32.21	30.40	0.118	0.302	0.960	0.273	7.38g	7.49	26.73	0.382	" "
29	18	32.80	1.21	2.52	30.32	28.75	0.126	0.293	1.12	0.28	7.43c	7.45	26.55	0.393	" "
5	20	35.32	1.21	4.65	29.24	27.87	0.136	0.325	1.41	0.25	7.48c	7.47	26.50	0.383	" "
26	18	40.36	1.21	2.52	38.68	36.39	0.135	0.508	1.61	0.29	7.36c	7.41	26.20	0.401	" "
20	68	40.36	1.21	2.52	38.06	36.60	0.242	0.397	2.42	0.17	7.61c	7.39	26.55	0.401	" "
15	8 days.	40.36	1.21	7.76	28.24	27.00	0.145	0.063	0.303	0.43	7.52c	7.21*	27.15	0.377	" "
25	18	40.36	1.21	2.52	38.07	35.73	0.127	0.677	2.04	0.19	7.34c	7.62*	26.54	0.401	CO_3 ppt. PO_4 ppt.
21	68	40.36	1.21	2.52	41.52	38.03	0.093	0.444	0.85	0.42	7.18c	7.41	26.28	0.402	" "
23	7 days.	30.27	2.42	None.	31.85	30.19	0.132	1.40	5.33	0.03	7.43c	8.40*	28.10*	0.379	CO_3 diss. PO_4 ppt.
												7.43	**26.53**		**Average of Groups IV and V.**

*Not figured in computing average.

meantime, $Ca_3(PO_4)_2$ dissolved in order to furnish $PO_4^{\equiv}$ ion to satisfy the $Ca_3(PO_4)_2$ stoichiometric solubility product. But in dissolving, it also furnished *more* Ca^{++} ions causing further precipitation of the carbonate and diminution of calcium. Bicarbonate was reduced, H_2CO_3 increased, and the solution became more acid. Apparently the reaction had not been completed in 20 hours, for a condition of slight supersaturation with respect to carbonate, and slight unsaturation with respect to phosphate, was obtained at the end of that period.

Group III. Experiments 16 and 24.—This group differs markedly from all of the others presented in this section in this respect: The solutions initially contained bicarbonate, carbonate, phosphate, but no calcium. In order to satisfy the equilibrium condition that the solution be saturated with respect to both solid phases, it was necessary that some Ca^{++} ion go into solution by dissolving $CaCO_3$ and $Ca_3(PO_4)_2$. The latter, furnishing $PO_4^{\equiv}$ ion to a solution already holding a large amount, dissolved but slightly, if at all. Hence $CaCO_3$ dissolved, furnishing HCO_3^-, and $CO_3^=$ ions, according to the reactions

$$(a)\quad CaCO_3 + H_2CO_3 \rightleftarrows Ca(HCO_3)_2 \rightleftarrows Ca^{++} + 2HCO_3^- \quad \begin{matrix}\uparrow\downarrow\\ 2H^+ + 2CO_3^=\end{matrix}$$

Also, $Ca_3(PO_4)_2$ may have reacted though slightly, as

$$(b)\quad Ca_3(PO_4)_2 + 2H_2CO_3 \rightleftarrows \underset{\begin{matrix}\uparrow\downarrow\\ 2Ca^{++} + 2HPO_4^=\\ \uparrow\downarrow\\ 2H^+ + 2PO_4^{\equiv}\end{matrix}}{2CaHPO_4} + \underset{\begin{matrix}\uparrow\downarrow\\ Ca^{++} + 2HCO_3^-\\ \uparrow\downarrow\\ 2H^+ + 2CO_3^=\end{matrix}}{Ca(HCO_3)_2}$$

Therefore reaction (*b*) was stopped with but little Ca^{++} in solution, and an increase in both $PO_4^{\equiv}$ and $CO_3^=$ ion, especially at slightly greater alkalinity ($p\alpha_{H^+}$ = 7.50 and 7.57). Since there was already sufficient $CO_3^=$ ion in solution to take care that $pK'_{s.p.}$ $CaCO_3$ was satisfied, the reaction (*a*) tended to the left. Since the solubility product of $Ca_3(PO_4)_2$ was greatly exceeded, reaction (*b*) also tended to run to the left, causing a decrease in $[Ca^{++}]$. However, such a decrease would have disturbed reaction (*a*) again, whereas the solubility product for $CaCO_3$ was almost at the equilibrium value. The rate of adjustment was apparently

* Not figured in computing average.

7.43 | 26.53 Average of Groups IV and V.

too slow, the final result being a supersaturation of $Ca_3(PO_4)_2$. It may be noted that the final state of affairs for Experiment 24 after 7 days saturation was very closely approximated by the final result of No. 16, a similar experiment, which had been run only for a little less than 2 days.

Group IV.—All of the experiments in this group had initially in solution, calcium, phosphate, and bicarbonate. Hence all reactions were precipitation reactions and should have been fairly well complete in a short time, in the presence of the two solid phases. Results of this and of Group V show an average of $pK'_{s.p.}CaCO_3$ = 7.43 at an average ionic strength for which $\sqrt{\mu} = 0.387$. The experiment upon $CaCO_3$ and $Ca_3(PO_4)_2$ solubility with changing concentration indicated a value of 7.46 for $pK'_{s.p.}CaCO_3$ at this ionic strength. The corresponding average $pK'_{s.p.}Ca_3(PO_4)_2$ found was 26.53, the actual dilution curve at this ionic strength being at 26.67. Undoubtedly, the experiments in this group represent the best ones of simultaneous calcium carbonate and phosphate equilibria. Under these conditions, equilibrium is rapidly attained.

Examination of the data for all experiments of the five groups reveals no apparent correlation between the stoichiometric solubility products obtained, and variation in $p\alpha_{H^+}$, final composition, or time of saturation after 16 hours. Hence these results were considered as a correct basis for comparison with results to be given on work with biological fluids.

Table IX gives the corresponding results of Holt, La Mer, and Chown (p. 542) recalculated on the basis of the stoichiometric dissociation constants of phosphoric acid used in this work. By comparison with our own, we find the following for $pK'_{s.p.}$ $Ca_3(PO_4)_2$:

Author.	Serum salts.	NaCl curve.	$\sqrt{\mu}$
Holt, La Mer, and Chown	25.58	27.75	0.382
Sendroy and Hastings	26.53	26.67	0.387

As pointed out in a previous section, our results with artificial edema fluid check those with NaCl solutions quite well for $pK'_{s.p.}$ $Ca_3(PO_4)_2$ and $pK_1''H_2CO_3$. However, all of these experiments

were performed under very similar conditions. Holt, La Mer, and Chown obtained serum salt solubility products for $Ca_3(PO_4)_2$ at $p\alpha_{H^+}$ between 6.95 and 7.70. In this respect, their experiments are comparable with ours, and the discrepancy between them may be ascribed to the difference in saturation. One might expect the velocity of precipitation of $Ca_3(PO_4)_2$ in their experiments to be somewhat less than if the reaction had taken place in the presence of solid $Ca_3(PO_4)_2$. On the other hand, the discrepancy between their NaCl dilution curve and ours, and between

TABLE IX.

$pK'_{s.p.}$ $Ca_3(PO_4)_2$ at 38° in Serum Salt Solutions.

Recalculated from Holt, La Mer, and Chown (1925,*a*), Table IX, p. 542.

$Ca(OH)_2+H_3PO_4$, shaking 8 days.

Experiment No.	$p\alpha_{H^+}$	$[Ca^{++}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[H_2PO_4^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HPO_4^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa^{++}	$pPO_4^{\equiv}$	$pK'_{s.p.}$ $Ca_3(PO_4)_2$	μ	$\sqrt{\mu}$
1	7.02	0.144	6.52	1.93	4.59	7.96	3.84	7.10	25.72	0.1487	0.386
2	6.95	0.126	6.36	2.15	4.21	6.22	3.90	7.21	26.12	0.1480	0.385
3	7.05	0.151	6.13	1.77	4.36	8.11	3.82	7.09	25.64	0.1493	0.386
4	7.05	0.124	5.91	1.71	4.20	7.82	3.91	7.11	25.95	0.1477	0.384
5	6.92	0.146	7.57	2.68	4.89	6.82	3.84	7.17	25.86	0.1462	0.382
6	7.22	0.154	4.55	0.98	3.57	9.82	3.81	7.01	25.45	0.1463	0.382
7	7.22	0.134	3.94	0.85	3.09	8.55	3.87	7.07	25.75	0.1451	0.381
8	7.29	0.096	3.23	0.61	2.62	8.50	4.02	7.07	26.20	0.1440	0.379
9	7.40	0.126	2.65	0.41	2.24	9.36	3.90	7.03	25.76	0.1431	0.378
10	7.10	0.154	4.45	1.18	3.27	6.83	3.81	7.17	25.77	0.1421	0.377
11	7.70	0.116	1.64	0.14	1.50	12.50	3.94	6.90	25.62	0.1410	0.376
Average									25.58		

it and their own serum salt experiments, is difficult to explain. It may be that our use of what may be uncertain values for the first or the third stoichiometric dissociation constant of phosphoric acid at such low $p\alpha_{H^+}$ results in the variation noted. The experiments on $Ca_3(PO_4)_2$ solubility alone in salt solutions, Paper II, all showed a higher figure for $pK'_{s.p.}Ca_3(PO_4)_2$. The reason for this is not clear. However, inasmuch as our interest is centered upon systems in equilibrium with both solids, it will be unnecessary to take into account the results of other systems at this time.

Solubility of Bone in Salt Solution.—In order to be able to draw conclusions as to the behavior of the bone salts from the foregoing experiments with solid $CaCO_3$ and $Ca_3(PO_4)_2$ *in vitro*, it was considered desirable to make certain that the carbonate and phosphate of bone behaved as did the isolated salts used in our experiments. Dried human femur bones were obtained and pulverized into a fine powder. The dried and decomposed organic matter, lipoids, etc., in the powder, was not separated in Experiments 25 and 21. In one experiment, No. 23, the material was thoroughly washed several times. Group V, Table VIII, gives the results obtained with artificial edema fluid when saturated with bone powder. Experiments 21 and 25 show little difference from the results obtained in Group IV. Experiment 23 seems to be very much like those of Table II, Paper II, where only calcium phosphate was used as the saturating body. It is not known whether the effect of thorough washing would cause the discrepancy noted, or not. One may conclude from these experiments, however, that the calcium salts of bone do not behave very differently from the inorganic salts $CaCO_3$ and $Ca_3(PO_4)_2$.

Solubility of Calcium Carbonate and Tertiary Calcium Phosphate in Serum.

In the previous section the conditions under which serum salt solutions could be brought into simultaneous equilibrium with $CaCO_3$ and $Ca_3(PO_4)_2$ have been described. Using the data so obtained, it was thought that we were in a better position to understand, if not to explain, the problem of serum calcium and its relation to the bone calcium.

By studying the reaction of serum when subjected to widely varying changes in its composition and condition, and by comparing its reaction to such changes with its normal behavior, an effort was made to arrive at a satisfactory explanation of the nature of the serum calcium and the manner in which it exercises its physiological function.

In such a study, we must realize that when one is dealing with serum it may or may not exhibit *in vitro* all or some of the properties that it possesses when in the body where it has the opportunity to react with other tissues. Nevertheless, realizing this limitation, on the basis of our experiments, we have been able to

arrive at some definite conclusions as to the nature of the calcium compounds of serum. The behavior of these compounds affords a reasonable, though only partial explanation of the physiological behavior of serum as a medium for transporting calcium.

Serum as drawn at normal $p\alpha_{H^+}$ had values of $p([Ca] \times [CO_3^{=}])$ or $pCaCO_3$ of 6.40, and of $p([Ca]^3 \times [PO_4^{\equiv}]^2)$ or $pCa_3(PO_4)_2$, of 22.50. After saturation with solid $CaCO_3$ at 38°, these values did not change for $p\alpha_{H^+} = 7.40$. In fact, there had been practically no change in total [Ca] or total $[PO_4]$ over a wide $p\alpha_{H^+}$ range. After saturation with solid $Ca_3(PO_4)_2$, however, $pCaCO_3$ was 7.23 and $pCa_3(PO_4)_2$ was 24.77 at $p\alpha_{H^+}$ 7.37 (Table IV, Paper II). There had been a decided decrease in [Ca] from 3.19 to 0.62 mM per kilo of H_2O, while $[PO_4]$ had remained almost constant. All of these values are lower than those for $pK'_{s.p.}CaCO_3$ (7.40) and $pK'_{s.p.}$(26.50) in serum salt solutions.

From thermodynamic considerations, it has been shown that, when a solution is in equilibrium with a solid salt such as $CaCO_3$, *it is a necessity* that the product of the activity of Ca^{++} ion times the activity of the $CO_3^{=}$ ion be a constant,—which is the activity solubility product. In terms of concentration, at equilibrium at any given ionic strength, $[Ca^{++}] \times [CO_3^{=}] = K'_{s.p.}CaCO_3$. Hence, if the $[CO_3^{=}]$ is fixed and known, and the stoichiometric solubility product at the given ionic strength is known, one may calculate for a system at equilibrium, the value for $[Ca^{++}]$.

It has been shown by Van Slyke and collaborators (1925) that the ionic strength of horse serum is about 0.167, when protein and other unknown anions are taken into consideration. For our purpose, it will be convenient here to assume $\mu = 0.160$ as a round figure, for *all* sera, the possible effect of protein being neglected for the time being. On comparing the values for salt solutions at this ionic strength, with those for serum at $p\alpha_{H^+} = 7.4$, we find in round numbers, at 38°,

(*a*) Salt solutions + $CaCO_3$, $pK'_{s.p.}$ $CaCO_3$ ..... = 7.4
(*b*) Serum as drawn, p $CaCO_3$ ..... = 6.4
(*c*) " + $CaCO_3$, p $CaCO_3$ ..... = 6.4
(*d*) Salt solutions + $Ca_3(PO_4)_2$ + $CaCO_3$ } $pK'_{s.p.}$ $Ca_3(PO_4)_2$ ..... = 26.5
(*e*) Serum as drawn, p $Ca_3(PO_4)_2$ ..... = 22.5
(*f*) " + $Ca_3(PO_4)_2$, p $Ca_3(PO_4)_2$ ..... = 24.7

XUM

Evidently, $pCaCO_3$ or $p([Ca] \times [CO_3^{=}])$ and $pCa_3(PO_4)_2$ or $p([Ca]^3 \times [PO_4^{\equiv}]^2)$ do not have the same signifiance in salt solutions as in serum, even when the latter is in equilibrium with the solid salt. This can only mean that at equilibrium of systems (*c*) and (*f*), all of the [Ca] is not in the form of *Ca^{++} ions*. Of course, for serum as drawn (*b*), the same conclusion would apply.

In arriving at this conclusion, several assumptions have been involved. The first is that the systems studied in the presence of the solid salts did come to equilibrium with the salt, regardless of what happened to the concentration of total calcium [Ca]. Even in the case of salt solutions containing phosphate which seemed to give high values for the solubility product of $CaCO_3$ in the presence of that salt, we are consistent with the above assumption, by assuming that the extra calcium was present, with phosphate in some form or other, *not* ionized. Thermodynamic considerations such as those on which this work is based make it extremely unlikely that the concentration of calcium ion in such a solution did not obey the rule expressed by equation (8) when the solid salt $CaCO_3$ was present. The criterion of equilibrium was the attainment of a constant value for $pK'_{s.p.}$. The minimum time for this under these conditions was not determined, but we are fairly certain that for most reactions our experiments were run long enough to rule this out as a factor.

Another assumption is that the activity coefficient of HCO_3^- and of $CO_3^{=}$ ion is the same in serum as in salt solutions. On the latter point, we have no data or direct evidence. However, as has been mentioned before, the activity coefficient of HCO_3^- ion in serum has already been found to be apparently almost identical with the same quantity in a salt solution of corresponding ionic strength. In the light of this, the assumption made with regard to $CO_3^{=}$ ion, does not seem an unreasonable one.

With these assumptions, one may proceed further. In such systems as may be considered in equilibrium with the solid, after having obtained the value for $[Ca^{++}]$, one may obtain [CaX] where, as has been mentioned before, [CaX] = total [Ca] − $[Ca^{++}]$. This does not, in any case, involve any explanation or assumption as to what CaX really is, other than that it is calcium apparently in solution not in the form of Ca^{++} ions.

Horse Serum Shaken with $CaCO_3$ and $Ca_3(PO_4)_2$ for Varying

XUM

TABLE X.

Effect of Shaking Horse Serum at 38° for Varying Lengths of Time, with Solid $CaCO_3$ and $Ca_3(PO_4)_2$.

Time.	p_{CH}+ (colorimetric).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^6}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa	$pCO_3^=$	$pCaCO_3$	Total $[PO_4]$ $\frac{M \times 10^6}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^6}{kg.\ H_2O}$	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$
0	7.46	29.68	28.21	0.132	3.19	2.50	3.88	6.38	0.79	3.25	7.49	22.48
5 min.	7.42	28.23	26.73	0.114	1.81	2.74	3.94	6.68	0.65	2.40	7.62	23.46
1 hr.	7.45	28.33	26.92	0.123	1.67	2.78	3.91	6.69	0.60	2.42	7.61	23.57
3 hrs.	7.45	27.95	26.56	0.121	1.67	2.78	3.92	6.70	0.59	2.37	7.63	23.59
22 "	7.23	22.03	20.37	0.056	1.49	2.83	4.25	7.08	0.35	0.77	8.11	24.72

Lengths of Time.—Although previous work showed that salt solutions could be placed in contact and brought into equilibrium with both solid $CaCO_3$ and $Ca_3(PO_4)_2$ by shaking or rotating for about 20 hours, it was thought desirable to attempt to verify this for serum. Holt, La Mer, and Chown, using sterile technique, shook serum with $Ca_3(PO_4)_2$ for from 8 to 10 days. Not only was this inconvenient, but such a procedure ran the risk of having some change, *e.g.* autolysis, occur in the serum thereby changing its character. If conditions could be adjusted so that the reaction was practically complete after 1 day, it would be a decided advantage. The results could then be compared with those of

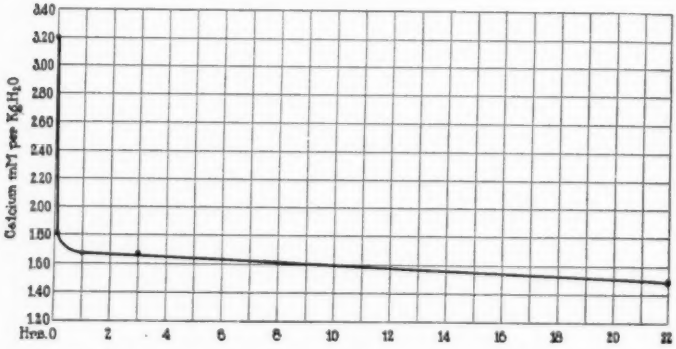

FIG. 4. The rate of precipitation of calcium from separated serum when it is saturated with solid $CaCO_3$ and $Ca_3(PO_4)_2$. Calcium in millimols per kilo of H_2O is plotted as ordinates and time in hours as abscissæ.

salt solution experiments performed under similar conditions. Furthermore, a study of the rate of change of calcium concentration would afford some indication of the mechanism of the reaction.

Accordingly, fresh horse serum from defibrinated blood was rotated at 38° with solid $CaCO_3$ and $Ca_3(PO_4)_2$; samples were removed at various intervals with the proper precautions and analyzed. Results are given in Table X and Fig. 4.

In contrast to the behavior of serum when saturated with only $CaCO_3$ as the solid phase, the total calcium decreased rapidly upon bringing the serum into contact with the solid phase. At first

sight, this would seem to be an argument for the theory of supersaturation with $Ca_3(PO_4)_2$. However, an examination of the change involved reveals that all of the calcium lost was precipitated not as phosphate, but mostly in the form of carbonate.

For example, in the first 3 hours of saturation there was a loss in bicarbonate and carbonate corresponding to the precipitation of 0.84 mM per kilo of H_2O of calcium as $CaCO_3$. The loss in total phosphate corresponded to the precipitation of 0.30 mM of calcium as $Ca_3(PO_4)_2$. By analysis, the actual loss in calcium was 1.52 mM, which is 0.38 mM more than can be accounted for from the decrease in bicarbonate, carbonate, and phosphate. From this experiment alone, one might infer that a condition of supersaturation with respect to both $CaCO_3$ and $Ca_3(PO_4)_2$ existed in the serum before equilibration with the solid.

This experiment is in agreement with the experimental results of Holt, La Mer, and Chown (see Table III, Paper II) on serum saturated with $Ca_3(PO_4)_2$, and with the results of Holt and his collaborators (1925, *b*), who saturated cerebrospinal fluid with $Ca_3(PO_4)_2$. These authors apparently overlooked the fact that the decrease in inorganic phosphate in their experiments was not sufficient to account for more than approximately 30 per cent of the decrease in calcium at $p\alpha_{H^+} = 7.40$. At greater alkalinity, more was lost as phosphate up to 52 per cent. It would seem, therefore, that a theory quantitatively accounting for the calcium in the blood must embrace other possibilities than only supersaturation with respect to $Ca_3(PO_4)_2$.

Does this experiment necessarily mean that serum is supersaturated with respect to $CaCO_3$? The experiments with only solid $CaCO_3$ would seem to be one argument against this possibility. It will be recalled that salt solutions containing phosphate after saturation with solid $CaCO_3$, still showed an apparent supersaturation with respect to $CaCO_3$. Upon adding $Ca_3(PO_4)_2$ also, a system was obtained, which soon attained equilibrium with both salts. The total calcium was decreased, but by theory, the Ca^{++} *ion* concentration in both systems before and after addition of $Ca_3(PO_4)_2$, must have been that demanded by equilibrium conditions.

Salt solutions containing sodium citrate showed what seemed to be a very high degree of supersaturation with respect to $CaCO_3$,

in the presence of the solid. Upon removing $CaCO_3$ and adding $Ca_3(PO_4)_2$ and saturating, some calcium was lost as the solution entered into equilibrium with the new phase. As a result, the system still showed a very high $pCaCO_3$ at a time when the solution was *unsaturated* with respect to $CaCO_3$.

Serum to which an excess of calcium had been added, retained this calcium and showed an apparent supersaturation of $CaCO_3$ even in the presence of the solid. Serum partially deprived of its calcium did not dissolve calcium on saturation with $CaCO_3$. On removal of phosphate, however, it was found possible to decrease or increase the total calcium even in the presence of solid $CaCO_3$.

In all of these conditions, the total [Ca] was always more than would be found in a simple salt solution of the same $[CO_3^{=}]$ after saturation with $CaCO_3$. Hence, it can be seen that in salt solutions it is possible under several different conditions for a system to be in equilibrium with solid $CaCO_3$, to show an apparent supersaturation with respect to $CaCO_3$, and to lose calcium upon being equilibrated with $Ca_3(PO_4)_2$. Hence, although $CaCO_3$ was precipitated from serum on adding solid $CaCO_3$ and solid $Ca_3(PO_4)_2$ in the light of its lack of response to $CaCO_3$ alone, we must conclude that its behavior in the case under consideration was due to the presence of the $Ca_3(PO_4)_2$ and its adjustment to the new solid phase, and not because it may have been supersaturated with respect to $CaCO_3$. It is highly improbable that any mechanical phenomena could have prevented the precipitation of $CaCO_3$ from a supersaturated solution in the presence of $CaCO_3$. Moreover, it is difficult to see how any such factor should have become inoperative on the addition of $Ca_3(PO_4)_2$.

Horse Blood Shaken with $CaCO_3$ and $Ca_3(PO_4)_2$ for Varying Lengths of Time.—In order to determine whether the presence of cells altered the reaction of serum toward solid $CaCO_3$ and $Ca_3(PO_4)_2$, freshly drawn defibrinated whole blood was brought into contact with both of these salts, samples were taken, and the serum separated and analyzed at various time intervals. It was anticipated that the presence of the cells might act to delay equilibrium, due to mechanical causes. When compared with the previous time experiment with separated serum, it is evident (Table XI, Fig. 5) that the cells exerted no appreciable influence

chemically or mechanically, on the final result with respect to $CaCO_3$. No analyses were made for phosphate. Inasmuch as the blood used here was not the same as that used for the serum experiment, a direct comparison cannot be made. The rate of precipitation of calcium, however, was apparently the same as for the previous experiment, most of the decrease taking place within the first 5 minutes. The zero point was assumed from other analyses of the same horse serum.

Effect of Shaking Untreated Horse Serum at 38° at Varying $p\alpha_{H^+}$.—In order to discover the effect on our systems, if any, of merely rotating serum at 38° for a period of about 16 hours, without any solid present, the experiment to be described was

TABLE XI.

Effect of Shaking Horse Blood at 38° for Varying Lengths of Time, with Solid $CaCO_3$ *and* $Ca_3(PO_4)_2$. *Serum Separated and Analyzed.*

Saturation time.	$p\alpha_{H^+}$ (colorimetric).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa	$pCO_3^=$	$pCaCO_3$
0	7.40*				3.30*	2.47	3.91*	6.38
5 min.	7.44	28.70	27.22	0.122	2.20	2.66	3.91	6.57
3 hrs.	7.38	26.59	25.09	0.098	1.49	2.83	4.01	6.84
20 "	7.32	22.50	21.09	0.071	1.31	2.88	4.15	7.03

* Assumed.

performed. Fresh horse serum was equilibrated at varying tensions of CO_2 to give the desired $p\alpha_{H^+}$ range. From the total CO_2 and the CO_2 tension $p\alpha_{H^+}$ was calculated from the curve for the ratio $\frac{pCO_2}{[CO_2]} = f_3$ at varying $p\alpha_{H^+}$, as described in the section on calculations. Results are recorded in Table XII.

The results indicate that shaking serum at 38°, in itself, has no effect on the amount of calcium held in solution, within a very wide range of $p\alpha_{H^+}$. Starting with a concentration of 3.40 mM Ca per kilo of H_2O, there was practically no change in this quantity from $p\alpha_{H^+}$ 6.27 to 7.95.

This experiment would suggest that whatever it is that keeps the calcium at a constant figure *in vivo* as far as change in $p\alpha_{H^+}$

is concerned, is still operative *in vitro*. Serum is apparently very well adapted to the biological function of keeping the calcium of the blood stream always at a constant level, within wide limits of variation of $[H^+]$, $[CO_3^{=}]$, and $[PO_4^{=}]$.

Experiments given in Paper I in which $CaCO_3$ was present, under the same conditions, gave almost the identical curve for $pCaCO_3$ and $pCa_3(PO_4)_2$ with varying $p\alpha_{H+}$, as was given by serum rotated without solid salts. The immediate inference would be that serum in the body is in equilibrium with $CaCO_3$. However, it is known that under normal conditions in the body,

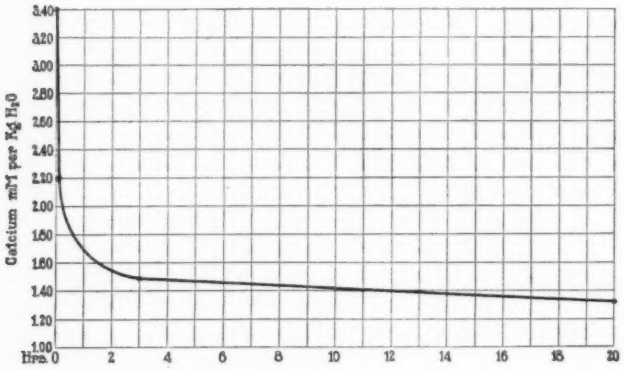

FIG. 5. The rate of precipitation of calcium from serum when whole blood is saturated with solid $CaCO_3$ and $Ca_3(PO_4)_2$. Calcium in millimols per kilo of H_2O is plotted as ordinates and time in hours as abscissæ.

the serum calcium is constant. Serum calcium *in vitro* has also been shown to be constant with change of $p\alpha_{H+}$, *when not in contact with* $CaCO_3$, and also to be unchanged when $CaCO_3$ was added. Hence, we cannot say that the serum was in equilibrium with $CaCO_3$ *in vivo* or *in vitro*.

Horse Serum Shaken at 38° with $CaCO_3$ and $Ca_3(PO_4)_2$ at Varying $p\alpha_{H+}$.—This experiment was performed in the same manner as the previous one without solids. The serum used had been standing in the ice box for a few hours before the experiment. The results obtained are given in Table XIII.

Here, the total calcium started with an initial concentration

TABLE XII.
Effect of Shaking Untreated Horse Serum at 38° for 16 Hours, at Varying $p\alpha_{H+}$.

TABLE XII.

Effect of Shaking Untreated Horse Serum at 38° for 16 Hours, at Varying $p\alpha_{H^+}$.

Experiment No.	$p\alpha_{H^+}$ (calculated).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCO_2 mm.	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa	$pCO_3^=$	$pCaCO_3$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$
1	6.27	56.35	711.9	32.72	0.00997	3.35	2.48	5.00	7.48	0.692	0.0623	9.21	25.85
2	6.92	38.53	160.6	33.15	0.045	3.40	2.47	4.35	6.82	0.766	0.690	8.16	23.73
3	7.55	30.81	34.3	29.50	0.170	3.40	2.47	3.77	6.24	0.762	4.0	7.40	22.21
4	7.95	27.48	12.3	26.70	0.371	3.40	2.47	3.43	5.90	0.759	10.63	6.97	21.36
5	8.40	23.51	3.67	22.47	0.915	3.06	2.51	3.04	5.55	0.620	25.4	6.60	20.72
Original.						3.40							

TABLE XIII.

Effect of Shaking Horse Serum for 17 Hours at 38°, with Solid $CaCO_3$ and $Ca_3(PO_4)_2$.

Experiment No.	$p\alpha_{H^+}$ (calculated).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCO_2 mm.	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCa	$pCO_3^=$	$pCaCO_3$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$
1	6.25	52.45	682.6	29.77	0.00865	3.04	2.52	5.06	7.58	0.57	0.0472	9.33	26.21
2	6.85	31.28	150.4	26.66	0.030	1.32	2.88	4.52	7.40	0.544	0.381	8.42	25.48
3	7.21	26.49	62.1	24.34	0.064	0.98	3.01	4.19	7.20	0.62	1.30	7.87	24.80
4	7.80	21.78	13.52	21.12	0.215	1.18	2.93	3.67	6.60	0.876	8.58	7.07	22.92
5	8.17	18.84	5.14	18.23	0.439	1.19	2.92	3.36	6.28	0.887	21.3	6.67	22.10
Original as drawn.	7.35c	13.01	52.7 (Calculated.)	29.15	0.106	3.30	2.48	3.97	6.45	0.91	2.82	7.55	22.54
Original before saturation.....						3.03							

for [Ca] of 3.03 mM. This was maintained at $p\alpha_{H^+}$ 6.25, but *decreased* to 0.98, increasing again between $p\alpha_{H^+}$ = 7.21 and 7.80. This latter increase may be ascribed to the base-binding power of the proteins, which increases with $p\alpha_{H^+}$ causing a decrease in [HCO_3^-]. At low $p\alpha_{H^+}$, phosphate was lost even when calcium was not.

The important point is, that there was no evident precipitation of calcium as $Ca_3(PO_4)_2$. Some HCO_3^- was lost; some of it by precipitation, some due to the increase in base-binding power of the proteins. One may conclude from this experiment that supersaturation with respect to $Ca_3(PO_4)_2$ does not explain the decrease in [Ca] when $Ca_3(PO_4)_2$ is added to serum. Hence, supersaturation is not the hypothesis which explains or accounts for the existence of CaX. That portion of CaX which is left after serum has been rotated with $CaCO_3$ and $Ca_3(PO_4)_2$ increases with the $p\alpha_{H^+}$. This is in harmony with what one finds in a salt solution containing serum proteins, and is the result of the increased calcium power of the serum proteins with increasing $p\alpha_{H^+}$.

Horse Serum with Added $Ca(HCO_3)_2$ Rotated at 38° with $CaCO_3$ and $Ca_3(PO_4)_2$ at Varying $p\alpha_{H^+}$.—Previous experiments with $CaCO_3$ showed that serum could retain apparently in solution an excess of calcium added as $CaCl_2$, even in the presence of solid $CaCO_3$. This was also true when the added calcium was in the form of $Ca(HCO_3)_2$. To test this property in the presence of both $CaCO_3$ and $Ca_3(PO_4)_2$ as solid phases, calcium chloride was added to serum with an equivalent amount of $NaHCO_3$ to make the [Ca] concentration 7.41 mM per kilo of H_2O—more than double the original calcium content. The results are given in Table XIV.

Although phosphate was precipitated, as might be expected, in the presence of the solid phase and so much extra calcium, the total calcium also fell markedly; it did not reach the level of the previous experiment at any corresponding pH value; both [Ca] and [HCO_3^-] were high. This may be interpreted as an argument for the theory that $CaCO_3$ is in colloidal suspension or is attached to the surface of the proteins, or may mean the formation of a calcium-carbono-X compound, although there is nothing in the CO_2 figure to indicate this. It will be noticed that the $pCa_3(PO_4)_2$ value reached was near the value for the quantity in the

TABLE XIV.

Effect of Shaking Horse Serum at 38° for 17 Hours with $CaCO_3$ and $Ca_3(PO_4)_2$ after Addition of $Ca(HCO_3)_2$.

Experiment No.	$p\alpha_{H^+}$ (calculated).	Total $[CO_2]$ M × 10^3 / kg. H_2O	pCO_2 mm.	$[HCO_3^-]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca]$ M × 10^3 / kg. H_2O	pCa	$pCO_3^=$	$pCaCO_3$	Total $[PO_4]$ M × 10^3 / kg. H_2O	$[PO_4^{\equiv}]$ M × 10^6 / kg. H_2O	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$
1	6.20	50.68	705.2	27.26	0.0071	5.40	2.27	5.15	7.42	0.362	0.0246	9.61	26.03
2	6.72	30.17	186.7	23.95	0.0204	4.91	2.31	4.69	7.00	0.164	0.0758	9.12	25.17
3	7.12	23.41	65.9	21.18	0.045	4.41	2.36	4.35	6.71	0.113	0.1831	8.74	24.55
4	7.52	21.37	25.4	20.42	0.110	3.01	2.52	3.96	6.48	0.154	0.739	8.13	23.82
Original.						3.30							
Reinforced.						7.41							

TABLE XV.

Effect of Shaking Horse Serum at 38° for 16 Hours with $CaCO_3$ and $Ca_3(PO_4)_2$ after Addition of Excess Phosphate.

Experiment No.	$p\alpha_{H^+}$ (calculated).	Total $[CO_2]$ M × 10^3 / kg. H_2O	pCO_2 mm.	$[HCO_3^-]$ M × 10^3 / kg. H_2O	$[CO_3^=]$ M × 10^3 / kg. H_2O	$[Ca]$ M × 10^3 / kg. H_2O	pCa	$pCO_3^=$	$pCaCO_3$	Total $[PO_4]$ M × 10^3 / kg. H_2O	$[PO_4^{\equiv}]$ M × 10^6 / kg. H_2O	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$
1	6.18	50.38	718.7	26.51	0.00655	3.21	2.49	5.18	7.67	1.34	0.0844	9.07	25.62
2	6.75	29.68	174.5	23.86	0.022	1.52	2.82	4.66	7.48	1.31	0.655	8.18	24.83
3	7.19	28.44	68.4	26.11	0.065	1.02	2.99	4.19	7.18	1.34	2.68	7.57	24.11
4	7.77	22.48	15.2	21.77	0.207	0.81	3.09	3.68	6.77	1.26	11.41	6.94	23.16
5	8.15	19.74	56.1	19.12	0.434	0.73	3.14	3.36	6.50	1.25	28.23	6.55	22.52

previous experiment, whereas $pCaCO_3$ was lower than normal for these conditions.

Horse Serum Rotated at 38° with $CaCO_3$ and $Ca_3(PO_4)_2$ after the Addition of Excess Phosphate.—To normal horse serum was added a mixture of KH_2PO_4-Na_2HPO_4 of $p\alpha_{H^+}$ = 7.40, sufficient to increase the phosphate content by 1.21 mM per kilo of H_2O. The normal horse serum value would be about 0.8 mM per kilo of H_2O. This serum was then rotated in contact with the two solid salts for 16 hours. The results are given in Table XV.

This experiment shows that calcium in serum is very easily precipitated in the presence of an excess of phosphate and the solid phase $Ca_3(PO_4)_2$. Moreover, examination of the phosphate figures reveals that although some phosphate had been precipitated, the amount in solution was almost constant at a high figure throughout a wide range of $p\alpha_{H^+}$, whereas during this same period the calcium dropped steadily. Some of it was evidently precipitated as $CaCO_3$ since the loss in PO_4 cannot account at any $p\alpha_{H^+}$ for the loss in calcium. In the presence of excess phosphate, the calcium precipitated at $p\alpha_{H^+}$ 7.4 to a point corresponding to the amount accounted for by the protein and salt content of the serum. That is, at $p\alpha_{H^+}$ 7.4, the [Ca] was the same as that of the experiment with two solids only.

By comparison with the previous experiment, it may be noted that the $pCaCO_3$ was lower in value throughout when excess $Ca(HCO_3)_2$ was present with the two solids, than when excess phosphate was added. The $pCa_3(PO_4)_2$ value for both conditions was very much the same. Hence some calcium phosphate must have been precipitated in the one case by excess calcium and in the other by excess phosphate. The carbonate concentration throughout being about the same for both experiments, the difference in calcium accounts for the difference in $pCaCO_3$ obtained.

This may possibly be regarded not as suggesting supersaturation with respect to $CaCO_3$ in the previous experiment, but as suggesting the presence of colloidal $CaCO_3$ in very fine particles in serum.

Horse Serum Rotated at 38° after Addition of Excess Phosphate.—This experiment, indicated in Table XVI, was similar to the one just described. PO_4 added was 2.25 mM per kilo of H_2O. However, no solid salts had been added. Under the circumstances,

TABLE XVI.

Effect of Shaking Horse Serum at 38° for 17 Hours after Addition of Excess Phosphate. No Solid Added.

Experiment No.	p_H+ (calculated).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	pCO_2 mm.	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	[Ca] $\frac{M \times 10^3}{kg.\ H_2O}$	pCa	$pCO_3=$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pPO_4=$	$pCa_3(PO_4)_2$	$pCaCO_3$
1	6.17	48.20	696.4	25.08	0.00626	3.30	2.48	5.20	3.00	0.18	8.75	24.97	7.68
2	6.87	38.69	178.4	33.06	0.039	3.24	2.49	4.41	3.04	2.28	7.64	22.75	6.90
3	7.49	29.63	38.0	28.23	0.141	3.15	2.50	3.85	2.99	13.31	6.88	21.25	6.35
4	7.94	25.21	11.4	24.50	0.328	2.56	2.59	3.48	2.73	37.1	6.43	20.63	6.07
5	8.32	21.94	4.10	21.08	0.724	2.56	2.59	3.14	2.67	90.7	6.04	19.85	5.73

reasoning by analogy from the experiments done on salt solutions, one would expect the condition of supersaturation shown here. This experiment, although it shows that serum can be apparently supersaturated with respect to calcium phosphate, does not constitute an argument for the hypothesis that *normal* serum in the body is supersaturated with respect to $Ca_3(PO_4)_2$. The content of phosphate in normal horse serum is such that it is but little precipitated when $Ca_3(PO_4)_2$ is added. Hence the extra calcium must be due to something else.

Variation of Calcium Concentration with Dilution of Serum after Equilibrating with $CaCO_3$ and $Ca_3(PO_4)_2$.—In order to show that that portion of calcium which was left after precipitating calcium from serum, by shaking with $CaCO_3$ and $Ca_3(PO_4)_2$, was of a definite composition, the following experiment was performed. Five solutions were prepared in which the serum was diluted with a balanced salt solution, so that the concentration varied from that of 100 per cent serum to 100 per cent salt solution. This salt solution was of an ionic strength $\sqrt{\mu} = 0.398$ and contained $NaHCO_3$, NaCl, KCl, $CaCl_2$, $MgCl_2$, K_2SO_4, and Na_2HPO_4-KH_2PO_4 in the proportions generally found in serum. Hence it was the proteins and constituents of serum other than the salts, which were being diluted. Samples were saturated with the solid phases for 17 hours, then analyzed.

Due to the dilution of the serum proteins, $p\alpha_{H^+}$ was found more accurately for Experiments 3 and 4 by calculation on the basis of analyses of CO_2 content and CO_2 tension. By using the proper $\alpha^o_{CO_2}$ in accordance with the formula $\alpha^o_{CO_2} = 0.557 - 103\,\mu + 0.36\,P$, and assuming a protein content of 7 per cent in (100 per cent) serum, $[H_2CO_3]$ was calculated. From this value, and the total $[CO_2]$, the factor $f_2 = \frac{[H_2CO_3]}{[CO_2]}$ was obtained from which the $p\alpha_{H^+}$ was calculated.

Values of 6.13 and 9.79 for pK_1'' and pK_2' respectively, were used throughout.

By taking into account the water content of the solutions, the protein percentage per kilo of H_2O was calculated. Table XVII gives the results of this experiment, and indicates that the total calcium is proportional to the amount of serum. The results are not strictly comparable due to differences in $p\alpha_{H^+}$.

This seems to prove that that portion of calcium which is still left, at least, has a definite composition. As has been pointed out, this is probably a calcium-protein compound.

These results agree with previous experiments in which serum was so diluted and saturated with $CaCO_3$. Other experiments in which the amount of dialyzed serum protein was varied, also showed a proportionality of serum protein to calcium.

Variation in Vitro of $pCaCO_3$ and $pCa_3(PO_4)_2$ with $p\alpha_{H^+}$ in Serum of Normal and Pathological Humans.—All of our *in vitro* experiments having been performed on horse serum, it was thought interesting to compare some results with the serum of normal and pathological human individuals. Table XVIII and Figs. 6 and 7 indicate the results obtained by varying the $p\alpha_{H^+}$

TABLE XVII.

Variation of Calcium with Varying Serum Concentration, after Shaking 17 Hours at 38°, with $CaCO_3$ and $Ca_3(PO_4)_2$.

Experiment No.	$p\alpha_{H^+}$	Total $[CO_3]$ $M \times 10^3$ / kg. H_2O	$[CO_3^=]$ $M \times 10^3$ / kg. H_2O	Total $[PO_4]$ $M \times 10^3$ / kg. H_2O	$[PO_4^{\equiv}]$ $M \times 10^3$ / kg. H_2O	[Ca] $M \times 10^3$ / kg. H_2O (observed.)	Per cent serum per kg. H_2O.
1	7.27c	28.61	0.080	0.394	0.98	1.30	100.0
2	7.38c	28.58	0.105	0.358	1.20	0.98	74.1
3	7.43g	28.70	0.119	0.384	1.46	0.63	48.7
4	7.44g	29.82	0.126	0.326	1.27	0.49	24.0
5	7.57c	30.07	0.154	0.250	1.21	0.26	0.0

of serum within physiological limits. No change, or very little, took place in either total phosphate or calcium, but bicarbonate as usual decreased with increase in $p\alpha_{H^+}$, due to the increase in base-binding power of the proteins.

The average value for $pCaCO_3$ at $p\alpha_{H^+}$ 7.40 was found to be 6.49, and for $pCa_3(PO_4)_2$, 22.42. The latter values are in agreement with those obtained on normal untreated horse serum at this $p\alpha_{H^+}$, and with Holt, La Mer, and Chown's values given in Table III, Paper II, before shaking with $Ca_3(PO_4)_2$.

The average decrease in $pCaCO_3$ with $p\alpha_{H^+}$ was found to be 0.90 between $p\alpha_{H^+}$ 7.2 and 7.5. This is really $\Delta pCO_3^=$ variation with $p\alpha_{H^+}$ and hence can be used to calculate the value of $pCaCO_3$ for untreated serum, from any determined $p\alpha_{H^+}$ to some set

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TABLE XVIII.

Variation in $pCaCO_3$ and $pCa_3(PO_4)_2$ of Serum in Vitro, with $p\alpha_{H^+}$. Human Serum of Normals and Pathological Cases.

Experiment No.	Condition.	$p\alpha_{H^+}$ (electrometric).	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^6}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^6}{kg.\ H_2O}$	pCa	$pCO_3^=$	$pCaCO_3$	$pPO_4^{\equiv}$	$pCa_3(PO_4)_2$	At $p\alpha_{H^+} = 7.40$ (calculated). $pCaCO_3$	At $p\alpha_{H^+} = 7.40$ (calculated). $pCa_3(PO_4)_2$
H1	Normal.	7.51	29.77	28.42	0.149	2.97			2.53	3.83	6.36			6.45	
H2		7.31	32.77	30.67	0.101	2.97			2.53	4.00	6.33				
O1	"	7.44	25.59	24.29	0.109	2.90	1.24	4.85	2.54	3.96	6.50	7.31	22.24	6.54	22.34
O2		7.23	28.25	26.11	0.072	2.85	1.24	2.77	2.54	4.14	6.69	7.56	22.77		
W1	"	7.50	30.83	29.42	0.151	2.63	1.29	5.90	2.58	3.82	6.40	7.23	22.20	6.49	22.43
W2		7.31	33.42	31.29	0.103	2.66	1.29	3.57	2.58	3.99	6.57	7.45	22.64		
H1	Pneumonia.	7.52	31.98	30.58	0.164	3.08	0.96	4.63	2.51	3.79	6.30	7.33	22.19	6.40	22.47
H2		7.32	34.63	32.46	0.110	3.08	0.96	2.74	2.51	3.96	6.47	7.56	22.65		
M1	"	7.49	29.23	27.90	0.140	2.98	1.29	5.77	2.53	3.85	6.38	7.24	22.07	6.46	22.26
M2		7.29	31.75	29.62	0.093	3.00	1.33	3.50	2.52	4.03	6.55	7.46	22.48		
G1	Cardiac.	7.55	33.22	31.83	0.183	2.72	1.31	6.83	2.57	3.74	6.31	7.17	22.05	6.45	22.41
G2	decompensation.	7.37	36.21	34.11	0.130	2.66	1.31	4.27	2.58	3.89	6.47	7.37	22.48		
V1	Chronic glomerular	7.51	29.53	28.20	0.148	2.44	1.30	6.11	2.61	3.83	6.44	7.21	22.25	6.55	22.52
V2	nephritis.	7.30	31.37	29.30	0.094	2.40	1.28	3.45	2.62	4.03	6.65	7.46	22.78		
C1	" "	7.55	30.33	29.07	0.167	2.35	1.34	6.98	2.63	3.78	6.41	7.16	22.21	6.55	22.55
C2		7.35	32.33	30.39	0.110	2.28	1.37	4.23	2.64	3.96	6.60	7.37	22.66		
Average														6.49	22.42
Range														6.40–6.55	22.26–22.55

reference $p\alpha_{H^+}$, as 7.40. Correspondingly, $\frac{\Delta pCa_3(PO_4)_2}{\Delta p\alpha_{H^+}}$ was found to be on the average – 2.35.

Since there was no solid phase present, we cannot calculate $[Ca^{++}]$ with any degree of certainty. However, if we *assume*

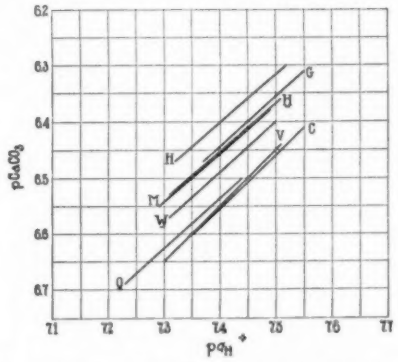

FIG. 6. The variation of $pCaCO_3$ with $p\alpha_{H^+}$ in normal and pathological humans. The letters designate the subjects. See Table XVIII.

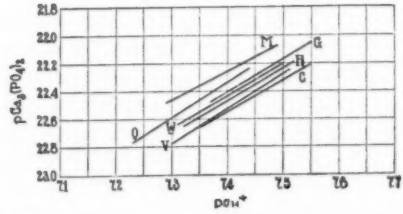

FIG. 7. The variation of $pCa_3(PO_4)_2$ with $p\alpha_{H^+}$ in normal and pathological humans. The letters designate the subjects. See Table XVIII.

equilibrium with $CaCO_3$, we find that at $p\alpha_{H^+} = 7.40$ the amount of calcium over and above the amount in solution in a salt solution of the same ionic strength and same $CO_3^=$ ion concentration would constitute about 85 per cent of the total calcium in these sera. Regardless of what this may be due to, it is an

index of the extent to which serum differs from a salt solution in its ability to transport the element calcium. The different levels of total calcium at the same $p\alpha_{H^+}$ may be ascribed to variation in any of the calcium-binding constituents of serum.

Solubility of Calcium Salts in Vivo in Serum of Pathological Humans.—It was thought interesting to determine whether one could form any conclusions as to the variation in calcium of the serum in various diseases. A few cases were studied. Blood was drawn under oil with proper precautions to prevent loss of CO_2, allowed to clot, and centrifuged within $\frac{1}{2}$ hour, then transferred to

TABLE XIX.

$pCaCO_3$ and $pCa_3(PO_4)_2$ in Pathological Serum as Drawn.

Subject.	Date.	$p\alpha_{H^+}$	Total $[CO_2]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[HCO_3^-]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[CO_3^=]$ $\frac{M \times 10^3}{kg.\ H_2O}$	Total $[PO_4]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[PO_4^{\equiv}]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$[Ca]$ $\frac{M \times 10^3}{kg.\ H_2O}$	$pCaCO_3$	$pCa_3(PO_4)_2$
C (Control.)	Jan. 11	7.34c	27.88	26.19	0.093	1.73	5.20	2.12	6.70	22.58
	" 12					1.71		2.38		
	" 13	7.23c	27.99	25.88	0.071	1.39	3.10	2.28	6.79	22.94
	" 14	7.23c				1.49	3.32	2.43		22.79
	" 16	7.35c	27.50	25.86	0.094	1.55	4.78	2.61	6.61	22.38
	" 19	7.34c	30.98	29.10	0.103	1.92	5.78	3.35	6.46	21.92
J		7.11	9.59	8.67	0.018	7.92	12.51	1.94	7.46	21.94
O		7.42	25.80	24.43	0.104	2.34	8.68	2.23	6.63	22.07
F		7.33	23.49	22.03	0.076	1.93	5.65	2.20	6.78	22.48
M		7.22e	15.14	13.97	0.038	3.50	7.59	1.45	7.26	22.76

containers over mercury. Analyses were made for $p\alpha_{H^+}$, CO_2, Ca, and total PO_4. The results are given in Table XIX.

A most interesting case is that of Subject J (chronic glomerular nephritis) who showed a very low bicarbonate, very high phosphate, and slightly low calcium in serum. Analysis would indicate that the calcium was on the salt level ($pCaCO_3 = 7.46$) none being bound by protein or the hypothetical X. However, a calculation of $pCa_3(PO_4)_2$ on the basis of the phosphate content would indicate a very high value for CaX.

If CaX were due to supersaturation of $Ca_3(PO_4)_2$ or $CaHPO_4$ or some form of calcium combined with phosphate, the real value of $p([Ca] \times [CO_3^=])$ would be even greater, indicating a high degree

of unsaturation for $CaCO_3$. However, not knowing the Ca^{++} ion concentration, we have but little indication of what has taken place in a case like this.

The serum *in vivo* was unable to make any quick response to the disturbed $CaCO_3$ equilibrium. In this case, not only was there a diminution in calcium proteinate, and perhaps CaX, but the symptoms of convulsions, indicating a disturbed condition of nerve and muscle irritability, suggested that the Ca^{++} ion concentration had decreased, the mechanism whereby the calcium is kept constant having failed to make the proper adjustments.

Table XIX also contains the results of the study of a case of glomerular nephritis (Subject C) made at this hospital in collaboration with Dr. J. F. McIntosh. The patient was treated daily for 1 week with parathyroid hormone injections, in an effort to relieve a persistent edema. No relief was obtained, but the results proved to be interesting from the standpoint of the therapy involved. During the injections there was a fall in $p\alpha_{H^+}$, a progressive increase in calcium, while phosphate remained persistently high. It is evident that the hormone caused an increase in total calcium. A change occurred in blood, whereby the factor which we call CaX was probably increased.

DISCUSSION.

In presenting the evidence, we have shown that the theory of complete dissociation of electrolytes, on the basis of the Debye-Hückel theory, seems to fail as an adequate explanation for the solubility of calcium in serum. If this theory were to serve, it would be necessary to show that serum was supersaturated with calcium carbonate, or calcium phosphate, or both; or that the activity coefficients of calcium carbonate and of calcium phosphate are lower in serum than those found to obtain in serum salt solutions.

In our calculations, we have assumed that the activity coefficient of $CO_3^{=}$ ion is the same in serum as in salt solutions. It has been pointed out that there is no direct evidence for this. In the same way, the activity coefficient of Ca^{++} ion may be different in serum from what it is in salt solutions. However, even if we were to assume an effect of serum on the activity coefficients of Ca^{++}, $CO_3^{=}$, and $PO_4^{\equiv}$ ions to be identical, we would still not obtain values

for $\gamma_{CO_3^=}$, $\gamma_{PO_4^=}$ and $\gamma_{Ca^{++}}$ which would explain the facts. Apparently, γ_{CaCO_3} would be 0.08 in serum instead of 0.26. We have no evidence at the present time, although such evidence may be produced in the future, that such specific ion effects may be attributed to the serum proteins.

Evidence has been produced which suggests that some portion of the serum calcium is bound by the proteins. However, in view of our lack of knowledge of the possible effect of the ionic strength of proteins in serum on the activity coefficients of the ions of difficultly soluble salts, we may only suggest what seems to be the probable magnitude of the calcium existing in the form of a protein compound. Apparently, this quantity is the amount that is left after saturating normal serum with solid calcium carbonate and tertiary calcium phosphate. It corresponds to a certain portion of what we call calcium unknown (CaX) in serum as drawn. Concerning the hypothesis of supersaturation, it may be stated that although such a condition may artificially be produced in salt solutions and in serum, the evidence in every direction seems to be strongly opposed to this point of view; at least, as the sole explanation for the apparently abnormal amount of calcium in serum.

Certainly, the experiments with $CaCO_3$ show no evidence of supersaturation. It has been shown that what apparent supersaturation of $Ca_3(PO_4)_2$ does sometimes occur, falls far short of accounting for the amount of extra calcium that exists apparently in solution in serum. In view of the experiments of Holt on cerebrospinal fluid saturated with $Ca_3(PO_4)_2$ and of others, the evidence seems strongly in favor of a diffusible, slightly ionized calcium compound. Our experiments with citrate certainly indicate that such a substance could behave the way serum calcium does. The change of $pCaCO_3$ and the change of $pCa_3(PO_4)_2$ with $p\alpha_{H^+}$ and with serum concentration, are points in favor of this hypothesis.

Although the evidence of experiments with $Ca_3(PO_4)_2$ and with $CaCO_3$ and $Ca_3(PO_4)_2$ apparently shows that the fall in calcium is due to the presence of $Ca_3(PO_4)_2$, we can only conclude, from the data, that the sole explanation is not supersaturation. As to exactly what happens when such large amounts of calcium are precipitated, we have no idea. The nature of the substance that

releases calcium as readily as our time experiments indicate, is unknown, and may only be the subject of speculation. Perhaps it is some slightly dissociable phosphate compound which holds calcium in unionized form.

It has been interesting to note, in this connection, that we also possess, in the active principle of the parathyroid gland, a sub-

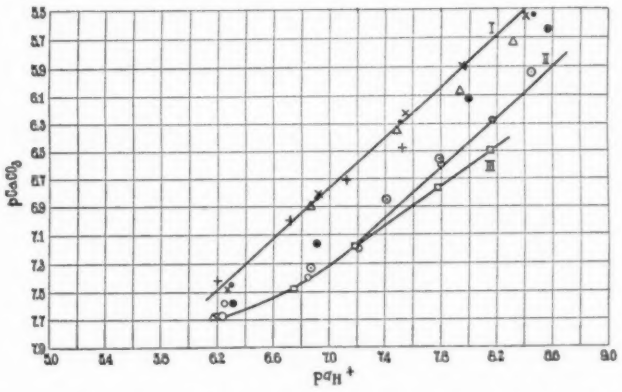

FIG. 8. The variation of $pCaCO_3$ with $p\alpha_{H^+}$ in horse serum under different experimental conditions. The points corresponding to the different experiments are designated as follows:

Symbol.	Experimental condition.
•	With solid $CaCO_3$. } Curve I.
×	Without any solid. }
○	With solid $CaCO_3$ and $Ca_3(PO_4)_2$. Curve II.
+	" excess Ca and HCO_3, and with solid $CaCO_3$ and $Ca_3(PO_4)_2$.
△	" " PO_4 " no solid.
□	" " " " with solid $CaCO_3$ and $Ca_3(PO_4)_2$. Curve III.
⊙	" solid $CaCO_3$ after depletion of calcium in solution.
●	" " " and parathormone after depletion of calcium in solution.

stance of biological origin, which can apparently hold calcium in solution, in much the same manner as do citrate solutions. It is highly improbable, however, that it can accomplish this alone and unassisted, in the body.

Concerning the question of equilibrium, it may be stated that the conditions for it have been obtained in our salt solution experi-

ments, and verified in those with serum at least with respect to $Ca_3(PO_4)_2$. Taking into consideration, the work of Holt, La Mer, and Chown on this question, together with our own evidence, it seems very improbable that any possible error in attainment of equilibrium could be of magnitude sufficiently great to affect any of the conclusions we have drawn.

The experiments presented, have, in large measure, been repeated often. Although they do not offer a complete solution to

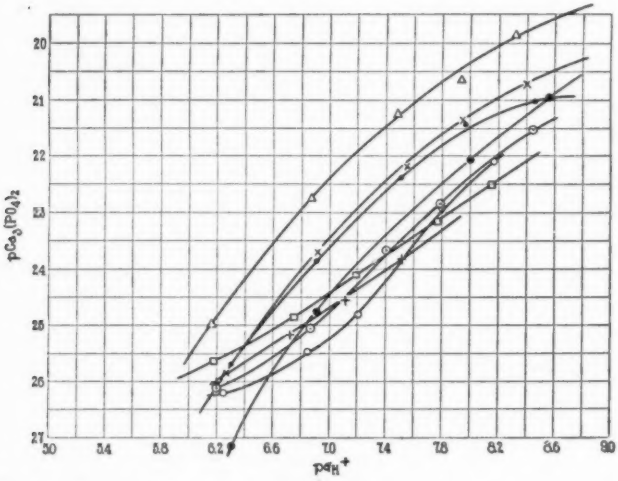

FIG. 9. The variation of $pCa_3(PO_4)_2$ with $p\alpha_{H^+}$ in horse serum under different experimental conditions. For explanation of symbols see legend to Fig. 8.

the problem of the nature of the calcium compounds of blood, the study of the solubility of the difficultly soluble salts has helped to increase our knowledge of the fitness of the mechanism which seems to be involved.

This work, together with what other evidence is available at the present time, indicates that we are dealing with a complex system adjusted to function, under normal conditions, in such a manner as to furnish the organism with the optimum quantity of calcium in ionized and unionized form, wherever and whenever it is needed.

The properties of serum, in this connection, seem to conform to the demands of such a system. While it is realized that we have but a small part of the physiological picture, inasmuch as most of our work has been done *in vitro*, our experiments are sufficiently clear in this respect; namely, that they indicate how calcium may be transported from one part of the body to the other.

For convenience, we have plotted in Figs. 8 and 9 the variation of $pCaCO_3$ and $pCa_3(PO_4)_2$ with $p\alpha_{H^+}$ under the various conditions for which they have been determined.

SUMMARY.

1. The solubility of $CaCO_3$ and $Ca_3(PO_4)_2$ in systems in equilibrium with both salts has been studied, and the conditions for equilibrium determined.

2. The stoichiometric solubility product of calcium carbonate has been found to vary with the ionic strength according to the equation $pK'_{s.p.}CaCO_3 = 8.58 - \frac{4.94\sqrt{\mu}}{1+1.85\sqrt{\mu}}$ at 38°.

3. The stoichiometric solubility product of tertiary calcium phosphate has been found to vary with the ionic strength according to the equation $pK'_{s.p.}Ca_3(PO_4)_2 = 30.95 - \frac{17.40\sqrt{\mu}}{1+1.48\sqrt{\mu}}$ at 38°.

4. The activity coefficient of Ca^{++} ion has been calculated from the solubility product data and the above equations.

5. The results indicate that these systems behave in conformity with the Debye-Hückel theory of strong electrolytes.

6. The solubility of calcium carbonate and tertiary calcium phosphate in balanced salt solutions under various conditions, has been determined.

7. The solubility of bone in salt solutions has been determined and found to be the same as that of its calcium salts.

8. The apparent solubility of calcium carbonate and tertiary calcium phosphate in serum has been studied under varying $p\alpha_{H^+}$ and time of saturation. Similar experiments without solids have been performed.

9. The solubility of these salts has been found to be unaffected *in vitro* by the presence of red blood cells.

10. The solubility of the salts has been found, after shaking,

with both salts, to be directly proportional to the serum concentration.

11. The variation *in vitro* of $pCaCO_3$ and $pCa_3(PO_4)_2$ with $p\alpha_{H+}$ in serum of normal and pathological humans has been studied.

12. The $pCaCO_3$ and $pCa_3(PO_4)_2$ of serum as drawn was determined in certain pathological cases.

13. The biological significance of these results has been discussed. The data indicate that the theory of supersaturation is inadequate; that calcium exists in serum in abnormal amounts bound to some substance or substances which hold it in solution in unionized form.

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